MOLECULAR APPROACHES TO THE IMPROVEMENT OF VIRAL SAFETY OF BLOOD AND BLOOD PRODUCTS

by

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DEDICATION

To my parents Ian and Margot.

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Detection of parvovirus B19 in blood and blood products. Scottish Virus Diagnostic Group meeting, City Hospital, Edinburgh, November 1991.

Detection and distribution of HCV genotypes in blood donors. Society for General Microbiology, Manchester, March 1993.

Distribution of hepatitis C virus (HCV) types 1-4 in blood donors from eight countries. Federation of European Microbiological Societies, Istanbul, Turkey, June/July 1993.

PCR, parvovirus and the testing of donor pools. British Blood Transfusion Society, Lancaster, September 1993.

HCV transmission by anti-D. Scottish Virus Diagnostic Group meeting, Heriot Watt University, Edinburgh, May 1994.

POSTER PRESENTATIONS

Mapping of serotype-specific, immunodominant epitopes in the NS-4 region of hepatitis C virus: use of type-specific peptides to serologically differentiate infection with HCV types 1, 2, and 3. Federation of European Microbiological Societies, Istanbul, Turkey, June/July 1993.

Geographical distribution of different hepatitis C virus types in blood donors: an international collaborative survey. IXth International Congress of Virology, Glasgow, August 1993.

ABSTRACT

This thesis is concerned with the detection of viruses in blood and blood products, with the general aim of improving the safety of blood donations and factor concentrates for clinical use. The prevention of virus transmission by blood and blood products is currently based on the screening of blood donations for virus specific antibody or antigen and by incorporation of virus inactivation procedures in the manufacturing process. In this study virus detection was by reverse transcription (RT) of virus RNA (where appropriate) followed by amplification of cDNA of virus DNA by the polymerase chain reaction (PCR). The viruses studied were hepatitis C virus (HCV), parvovirus B19 and hepatitis A virus (HAV).

For HCV, the relationship between detection of anti-HCV by recombinant immunoblot assay (RIBA-2) and viraemia was examined and a good correlation (84.4%) between a positive result in the RIBA-2 and detection of HCV by PCR was observed. However 5.4% of donations that were RIBA-2 indeterminate were PCR positive, demonstrating that PCR can be useful in the confirmation of ambiguous serological results. An assay system was developed that identifies different HCV genotypes by restriction fragment length polymorphisms (RFLP) in the 5′ non-coding region of the genome. In a worldwide study of the distribution of HCV genotypes in blood donors, two new genotypes were discovered. Distinct geographical distributions of HCV genotypes were observed; HCV types 1, 2, and 3 were widely distributed while genotypes 4, 5, and 6 were detected exclusively in donors from one country only. Donors infected with HCV type 1 showed broad serological reactivity with all

four antigens of the RIBA (5-1-1, c100-3, c33c, c22-3) while infection with other genotypes elicited antibody responses restricted to two antigens only (c33c and c22-3), irrespective of the geographical origin of the donor. This lack of cross-reactivity provides an explanation for the continued transmission of HCV by blood screened by assays relying on reactivity to c100-3 alone. Genotype determined by the RFLP typing assay correlated well with a type specific serological assay.

Transmission of HCV by intravenous immunoglobulin (IVIG) has been reported. In a retrospective study of a group of immunodeficient patients, HCV RNA was detected in patient plasma samples and some of the associated IVIG batches. Similarly, in a large group of rhesus negative women, HCV RNA was detected following administration of a specific batch of anti-D immunoglobulin after pregnancy. In this case, it was possible to show that the HCV variant in the IVIG preparation closely matched those in the samples obtained from recipients, indicating the value of direct nucleotide sequence comparison in molecular epidemiological studies.

For parvovirus B19 and HAV a method for screening a large number of blood donations was developed, and could be applied to prevent transfusion of these and other transfusion-transmission related viruses by exclusion of blood at source. B19 could be detected in batches of non-heat treated factor VIII and IX concentrate manufactured from plasma unscreened for B19 DNA. Dry-heat treatment reduced but did not eliminate detectable B19 from the product. Comparison of virus titre

established by PCR and by cell culture infectivity assays for both human immunodeficiency virus and canine parvovirus (an *in vitro* model for B19) demonstrated that virus DNA could still be detected in concentrates in which infective virus had been eliminated by heat treatment.

Overall, this work has shown the value of PCR in the screening of individual or pooled blood donations for the presence of viruses, in the identification of novel genotypes and in improving the safety of blood and blood products.

CHAPTER 1

1. INTRODUCTION

THE SAFETY OF BLOOD AND BLOOD PRODUCTS

The disease first known to be transmitted by blood and blood products was syphilis, which was also the first to be prevented by storage at low temperature. Parasitic diseases are also of concern. However they tend to be restricted to specific geographical areas while viral diseases are of universal concern. New infectious diseases are constantly arising which may be transmitted by blood and its products. A single unit of contaminated blood has the potential to infect many recipients. A unit of donated blood is routinely separated into red-cell concentrates, platelet concentrates and plasma. Human plasma in pools of 10,000 donations or more is used to manufacture a large range of therapeutic proteins (table 1.1.). The most frequently required are concentrates of Factor VIII and IX used in the treatment of haemophilia A and B respectively, and immunoglobulin preparations for intravenous infusion in treatment of patients with natural or induced immunodeficiency. Fractionation of contaminated plasma, combined with thousands of other donations ultimately yield products for a thousand different patients. Some infectious agents are more likely to be transmitted by the cellular components of blood while others may be transmitted by plasma and plasma products (table 1.2.). Protozoa and bacteria are the major contaminants of cellular blood components while viruses are the problem with plasma derivatives. Specific geographic areas constitute reservoirs with a high prevalence of certain infectious agents eg: donors from a malarial area are likely to transmit plasmodia, although it is quite probable that a recipient in such an area is likely to be infected anyway.

Table 1.1.
PLASMA PRODUCTS : INDICATIONS FOR USE

Plasma product concentrate	Indications for Use
Albumin	Volume expander
Human factor VIII complex	Congenital deficiency (haemophilia A: von-Willebrand's disease)
Factor IX prothrombin complex	Congenital deficiency (haemophilia B) Reversal of oral anticoagulant overdose Congenital deficiences of Factors II and X Factor VIII inhibitors Severe liver disease
Immunoglobulin	Passive prophylaxis Congenital agammagloblinaemia or Hypogamma-globulinaemia Types of immune thrombocytopenic purpura Other acquired immune disorders
Antithrombin III	Congenital deficiency Disseminated intravascular coagulation, liver transplantation, other acquired deficiency states
Factor IX	Congenital deficiency
Factor XIII	Congenital deficiency, fibrin sealant
Activated prothrombin complex	Factor VIII inhibitors
Protein C	Congenital deficiency
CI esterase inhibitor	Hereditary angioedema
Alpha-1 Antitrypsin	Hereditary deficiency (emphysema, cirrhosis)
Fibronectin	Acquired deficiency states, wound healing
Fibrinogen	Wound healing, tissue sealing

Table 1.2.

INFECTIOUS AGENTS TRANSMITTED BY BLOOD AND PLASMA PRODUCTS

Agent	Blood	Plasma and Plasma Product
Non-viral		
Bacteria	Yes	Occasionally
Protozoa	Yes	No
Viral		
Epstein-Barr	Yes	No
Cytomegalovirus	Yes	No
HTLV-I	Yes	Possible
HTLV-II	Unknown	Unknown
HIV	Yes	Yes
Hepatitis B	Yes	Yes
NANBH	Yes	Yes
Delta Agent	Yes	Yes
Parvovirus	Yes	Yes

1.1. VIRUSES TRANSMITTED BY BLOOD AND PLASMA PRODUCTS

As blood and plasma therapy developed during the Second World War it became clear that post-transfusion hepatitis was a "common" event in the USA (Beeson et al.,1943) resulting in programmes being introduced to reduce or remove the risk. Based on epidemiological characteristics and the incubation period after infusion of blood products, two clinical forms of hepatitis were defined (1) 'serum' hepatitis : associated with the use of blood and its products (now known to be hepatitis B); (2) 'infectious' hepatitis : associated with faecal-oral transmission and with a shorter incubation period (now termed hepatitis A). Introduction of screening for hepatitis B virus between 1970 and 1972 by detection of hepatitis B surface antigen (HBsAg) in association with the exclusive use of volunteer donations has reduced the incidence of carriers in the blood donating population to under 1 in 1000. With the introduction of HBsAg screening the frequency of post-transfusional hepatitis (PTH) has been reduced to less than 1% of hospitalised transfused recipients becoming infected in the UK. Although a few cases do occur they can be attributed to blood donors in whom the concentration of circulating HBsAg is below the levels of detection. Prior to the introduction of anti-HCV screening the majority of cases of PTH that occurred were attributed to non-A non-B hepatitis (NANBH), the causative agent is now known to be hepatitis C virus (HCV) (or group of viruses).

The number of viruses thought to be transmitted by blood products is quite limited. The reason whether a virus is likely to cause contamination of a plasma pool depends on the virus physicochemical properties and epidemiology. Viruses such as

cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human T-cell lymphotropic virus-I (HTLV-I) are not known to be transmitted by plasma products (Prince et al., 1987). Although blood-borne viruses, they are almost totally cell-associated and significant levels of cell-free virus are rarely found. Viruses such as hepatitis A (HAV) have a short-lived viraemia of 0 to 14 days, therefore the risk of a plasma donation being obtained during this period is quite low. However, recent reports (Mannucci 1992; Gerritzen et al., 1992; Normann et al., 1992) indicate that transmission of HAV by blood products does occur. Viruses transmitted by plasma products are usually those that cause persistent infection, also the frequency with which the virus is likely to cause active infection in the donating population needs to be taken into account with routes and frequency of transmission being important. Viruses that generate high plasma titres can remain at infective levels even following dilution in a plasma pool, while low viral titres present in a small number of donations are liable to be diluted to insignificant levels. To date, based on epidemiological data, only five human viruses have been shown to be transmitted:

(1) human immunodeficiency virus type I (HIV I)

(2) hepatitis B virus (HBV)

(3) hepatitis D virus (HDV)

(4) Non-A Non-B Hepatitis (NANBH or HCV)

(5) human parvovirus B19

although, as stated above, it is possible that other viruses have been transmitted by plasma products occasionally. The physicochemical properties of these viruses are

shown in table 1.3. and are relevant in consideration of inactivation procedures used in manufacturing processes.

Table 1.3.

PHYSICOCHEMICAL CHARACTERISTICS OF VIRUSES TRANSMITTED BY BLOOD AND PLASMA PRODUCTS

Virus	Diameter (nm)	Nucleic Acid	Lipid Envelope	Period of Viraemia	Virus Titre Attainable/ml
HIV-1	100-150	ss RNA	yes	long (yrs)	10^4-10^{10}
Hepatitis B	42	partially ds DNA	yes	3 months	10^7-10^8
Delta Virus*	35-37	ss RNA	no	4-12 weeks	10^{11}
Parvovirus B19	24	ss DNA	no	2-3 weeks	10^{12}
NANBH (HCV)∝	30-60	ssRNA	yes	long (yrs)	10^{12}

ss : single stranded
ds : double stranded
* Delta virus cannot replicate without the assistance of HBV. The delta genome cannot code for its own coat and uses the HBV envelope to provide protection for its RNA.
∝ Considering here only the parenterally transmitted non-A non-B hepatitis (NANBH), hepatitis C virus (HCV). Hepatitis E virus has been reported in tropical countries and is transmitted by the faecal-oral route. It is smaller, ~27-30nm and does not have a lipid envelope.

1.2. METHODS FOR REDUCING VIRUS LOAD IN PLASMA POOLS

At present there are two main approaches to improving the safety of blood and plasma pools prior to use or manufacture :

(1) careful selection of donors

(2) screening of donated units of blood

1.2.1. DONOR SELECTION

This approach relies on the selection of "healthy" donors using physical and laboratory techniques. Where possible donors are interviewed and questioned about history of hepatitis, intravenous drug abuse, homosexual activity or any other behaviour that would indicate a "high-risk" category. This approach has limited success as studies have shown that many blood donors who later developed AIDS were found to be homosexual and often drug addicts. In countries where donors are paid, such as the United States there is the added complication of the economic needs of the individual prompting him or her to give false answers when questioned. In addition the majority of carriers of hepatitis B have no history of hepatitis and therefore only a few carriers would be excluded by such a question.

1.2.2. SCREENING OF DONATIONS

Automated antibody screening tests are commercially available for selected infectious diseases. The use of microtitre plate formats enable reagents to be added to the test wells with ease either manually or mechanically. Results on several hundred samples can be obtained within a few hours of donation. Currently at the Scottish National

Blood Transfusion Service (SNBTS), donations are routinely screened for HBsAg and antibody to hepatitis C virus (HCV), human immunodeficiency virus (HIV) and syphilis.

1.3. INACTIVATION OF INFECTIOUS AGENTS IN COAGULATION FACTOR CONCENTRATES

The manufacturing method is of great importance since adverse reaction to the product may arise and the possibility of viral transmission by the preparation may occur. It is difficult to develop a manufacturing viral inactivation or removal procedure as any physical or chemical stage must not denature the plasma proteins. The European Community Note for Guidance states that " The virus reduction factor of an individual purification or inactivation step is defined as the $\log_{10}$ of the ratio of the virus load in the starting material and the virus load in the final material which is ready for use in the next step of the process" (Ad Hoc Working Party on Biotechnology/Pharmacy, 1991). Several methods are available for viral inactivation and different manufacturers favour different methods. A general overview of currently available techniques is shown in table 1.4 and those under development in table 1.5, immunoglobulin preparations are considered separately in chapter 5.

Up until 1991 heat treatment was the main method of virus inactivation used at the Protein Fractionation Centre (PFC) in Edinburgh. Experiments in which the temperature was raised from 68^0C to 80^0C in different product formulation showed

Table 1.4.

METHODS CURRENTLY USED FOR VIRAL INACTIVATION BY MANUFACTURERS OF PLASMA PRODUCTS

Method	Mechanism
Physical-Heat Treatment	Heating in solution Dry heat treatment Steam treatment of freeze-dried powder Heat/Solvent treatment of freeze-dried powder
Chemical	Solvent-Detergent Treatment β-Propiolactone/Ultraviolet Treatment

Table 1.5.

METHODS IN THE COURSE OF DEVELOPMENT FOR THE VIRAL INACTIVATION OF CELLULAR COMPONENTS, WHOLE BLOOD OR PLASMA

Method	Agent
Physical	Radiation: gamma,ultraviolet Ozone Washing with saline Filtration of leukocytes Pasteurisation
Photochemical	Psoralens Porphyrin derivatives Merocyanine 540 Methylene Blue
Chemical	Solvent and detergent Long Chain Fatty Acids Caprylate Sodium Chlorite

a significant increase of virus kill (Cuthbertson et al., unpublished data SNBTS). It is important to validate the heat/time combination used with infectivity studies fora particular product as varying reports have been published on the inactivation of HIV with different time/temperature regimes (McDougall et al., 1985; Tersmette et al., 1986; Prince, 1986a; Piszkiewicz et al., 1987; Levy et al., 1985; Reid et al., unpublished data SNBTS). Products developed in 1987 can be heated at 80^{0}C for 72 hours (a severe dry-heat treatment) and clinical results indicate no transmission of NANBH, HBV or HIV (Colvin et al., 1988; McIntosh et al., 1987). The major advantage of dry-heat treatment is that it is carried out in sealed containers as the final step in production, thus preventing the possibility of inadvertent contamination.

The presence of a lipid envelope on a virus particle renders it susceptible to inactivation by lipid solvents. The use of a nonvolatile solvent tri-(*n*-butyl) phosphate (TNBP), in combination with one or another of a range of detergents usually sodium deoxycholate (a natural constituent of the human gut) was first developed at the New York Blood Donor Centre (Prince et al., 1987) and is now used by many manufacturers worldwide. The TNBP/detergent treatment is carried out at 20 - 30^{0}C as the rate of viral inactivation is temperature dependent and is much slower at 4^{0}C. At the end of the inactivation step the solvent/detergent mixture is removed either by precipitation of the product or by chromatography. Virus inactivation studied by *in vitro* models have shown that the method is very effective for inactivation of lipid enveloped viruses, as expected. No cases of transmission of NANBH or HIV have been reported with this product, however the non-lipid enveloped

encephalomyocarditis virus (EMC) has been shown to be completely resistant to all TNBP/detergent treatment combinations. Although there have been no reports on parvovirus transmission it is unlikely that this virus would be inactivated by such a procedure due to a lack of a lipid envelope.

1.3.1. ASSESSMENT OF VIRUS INACTIVATION

In order to assess the manufacturing process it is necessary to have appropriate validation tests for the inactivation methods. Tissue culture (*in vitro*) studies are commonly used to demonstrate the adequate kill of HIV. The advisory committee in the U.K. (UK BTS/NIBSC Liaison Group) recommends that each manufacturer provides data demonstrating that one single step in the manufacturing process is capable of inactivating 5 logs of HIV-1 (Thomas, 1988). The laboratory-scale validation of HIV inactivation must simulate the full-scale process as closely as possible, so that the data is representative of the potential of large-scale manufacture. However at present there is no reliable culture system for the agents HBV, NANBH (HCV), HDV and parvovirus B19. Most manufactures make use of "model viruses" which include DNA and RNA viruses, with representatives of both enveloped and non-enveloped viruses, commonly used "models" are vaccinia - a lipid-enveloped, double-stranded DNA virus, and Semliki Forest Virus (SLFV) - a lipid-enveloped, single-stranded RNA virus. Problems arise with agents such as HCV as this has yet to be assign to a family, and shows similarity to both flaviviruses and togaviruses. Recent infectivity studies using the two flaviviruses, yellow fever virus (YFV) and tick-borne encephalitis virus (TBEV), and one pestivirus bovine diarrhoea virus

(BVDV) showed that all three viruses were inactivated after 6 hours heat treatment in aqueous solution at 60^{0}C. The physicochemical properties of HCV suggests that this virus is more closely related to the pestivirus group than the flavivirus group which would indicate that BVDV would be the appropriate virus for use in routine *in vitro* virus safety studies (Nowak et al., 1992). The most reliable model system for parvovirus B19 is the canine parvovirus (CPV) which can be cultured readily.

Animal (chimpanzee) studies are used by some manufactures as they are the only means of demonstrating the inactivation of HBV and HCV. However correlation of non-infectivity in chimpanzees with that in clinical evaluation have proven to be poor (Colombo et al., 1985; Kernoff et al., 1987). In addition the moral dilemma of using an endangered species such as the chimpanzee for a test that supplies limited information must also be considered.

Clinical (human) studies rely on the regular assessment of the serostatus of treated patients with any seroconversions traced to a particular product and/or batch. HBsAg status can be monitored indicating any HBV infection, while the seroconversion to HIV and parvovirus B19 can also be detected. Detection of abnormal liver enzyme levels (usually alanine aminotransferase - ALT) in combination with anti-HBc has until recently been the only available predictive monitor of NANBH infection. However most haemophiliacs have already been infected with NANBH, the only recognised method for assessing the risk of NANBH transmission is to study ALT levels in previously untreated patients (PUPs) following their initial infusions of

coagulation factor concentrates. This presents problems as the elevation in ALT levels may be transient so sampling frequency and times may be critical. However the advent of second and third generation enzyme-linked immunoassays (EIAs) and recombinant immunoblot assays (RIBA) which reliably detect HCV seroconversion have certainly overcome the need for ALT testing as the EIA is available for mass screening of donated blood with repeatedly reactive samples being tested by the supplemental test, the RIBA.

1.4. AIMS

The aim of the work described in this thesis was to provide a contribution to improving the safety of blood and blood products with respect to the transmission of viruses. One approach was at the level of donor screening, for HCV the results of antibody screening with the detection of viraemia were compared to assess the efficacy of currently available blood donor screening tests. This study was extended to investigate the effect of HCV sequence variation on the sensitivity and specificity of reaction in antibody tests. To overcome problems associated with antibody detection an alternative method would be to screen for viraemic donations on a routine basis. Parvovirus B19 and HAV were used as model viruses in the application and assessment of a pooling system for detection of viruses in blood donor screening. The role of PCR and sequence analysis to confirm cases and provide evidence for iatrogenic transmission of viruses by blood products was examined. Finally, the significance of the presence of viral nucleic acid in blood products was addressed.

CHAPTER 2

2. GENERAL METHODS

2.1. PLASMA SAMPLES, FACTOR CONCENTRATES AND INTRAVENOUS IMMUNOGLOBULIN PREPARATIONS

Plasma samples, factor concentrates and intravenous immunoglobulin preparations were made available from a wide range of sources and details are given in the appropriate sections. Blood donor plasma samples were frozen at either -20^{0}C or -40^{0}C as soon as practically possible in all studies. Freeze-dried preparations of Factor VIII and IX and intravenous immunoglobulin were stored at 4^{0}C prior to reconstitution. Once reconstituted aliquotes were removed and subjected to nucleic acid extraction immediately. The remaining product was stored at -20^{0}C. Lyophilised vials were reconstituted as specified by the manufacturer, 11 and 36 U/ml for factor VIII and IX respectively, and 50g/litre for intravenous immunoglobulin.

2.2. DNA EXTRACTION

The work carried out in this project required the extraction of nucleic acid from a large number of samples. The initial procedures used involved different protocols for the purification of DNA and RNA, in the second half of the work a new highly efficient extraction method was used which purified nucleic acid with equal efficiency for RNA and DNA (see section 2.4.). The DNA extraction procedure described in this section represents a reasonable compromise between quality of DNA and ease of use, yielding DNA in a reasonable time scale.

DNA was extracted from 0.5ml of plasma, by ultracentrifugation. The plasma sample was mixed with 6mls of RPMI medium (pH 7.3) in a polycarbonate centrifuge tube and sealed with a noryl cap assembly. Virus was pelleted at 100000 X g for 2 hours at 4^0C in a fixed angle rotor (rotor: Beckman Ti70; centrifuge: Beckman Model L8M). For Factor VIII and IX concentrates, intravenous immunoglobulin and plasma pools, 2.5mls of reconstituted product or plasma pool, was mixed with 4.0mls of RPMI medium and ultracentrifuged as described. Supernatant was removed using a pastette to ensure that the pellet was not disturbed, the pellet was resuspended in 400µl of lysis buffer: 50mM Tris-HCl pH8.0; 50mM EDTA pH8.0; 100mM NaCl; 0.01% w/v Proteinase K; 1% N-laurylsarcosine) and incubated at 65^0C for 2 hours. The pellet was then transferred to a 1.5ml eppendorf tube and extracted twice with 600µl of phenol and once with chloroform. The DNA was precipitated overnight in two volumes of ethanol at -20^0C. DNA was pelleted by centrifugation at 18000 X g for 15 minutes at 4^0C, the supernatant removed carefully and the pellet dried at 42^0C for 10 minutes, DNA was solubilised in 20 to 200µl of distilled water.

2.3. RNA EXTRACTION

In preparation of RNA considerable care must be taken to avoid degradation of the preparation by exogenous RNAses which may be present *in vitro* and *in vivo*. Virus was pelleted by ultracentrifugation as described in section 2.2.1. The pellet was resuspended in 1.0ml of a denaturing solution (2M guanidine thiocyanate; 12.5 mM sodium citrate pH7.0; 0.25% sarkosyl; 0.05M 2-mercaptoethanol; 50% water-

saturated distilled phenol). The solution was then transferred to a 1.5ml eppendorf microfuge tube and 200µl of chloroform added and mixed thoroughly using a vortimixer (Chomczynski et al., 1987). The solution was incubated at 4°C for 15 minutes then spun at 18000 X g for 10 minutes, the aqueous phase was removed into an equal volume of chloroform and mixed thoroughly as before, followed by further centrifugation to resolve the aqueous phase from the organic phase. The resulting aqueous phase was mixed with an equal volume of isopropanol and left at -20°C overnight to precipitate viral nucleic acid.

When the sample volume was 200µl or less a direct extraction was carried out. The plasma was added directly to the denaturing solution and mixed vigorously immediately. The extraction procedure was then carried out as described above.

Precipitated RNA was pelleted by centrifugation for 15 minutes at 18000 X g at 4°C. The pellet was washed once with 1ml of 75% ethanol and dried at 42°C for 10 minutes. The pellet was then dissolved in 20µl of pyrogen-free water treated with diethylpyrocarbonate (DEPC dH_2O).

2.4. IMPROVED EXTRACTION METHOD FOR NUCLEIC ACID

Either 100µl of plasma was extracted directly or 500µl was spun as described in section 2.2. and the pellet resuspended in the lysis buffer. (It was found that direct extraction of a volume larger than 100µl of serum or plasma had an inhibitory effect, probably due to there being insufficient proteinase K to inactivate proteins present).

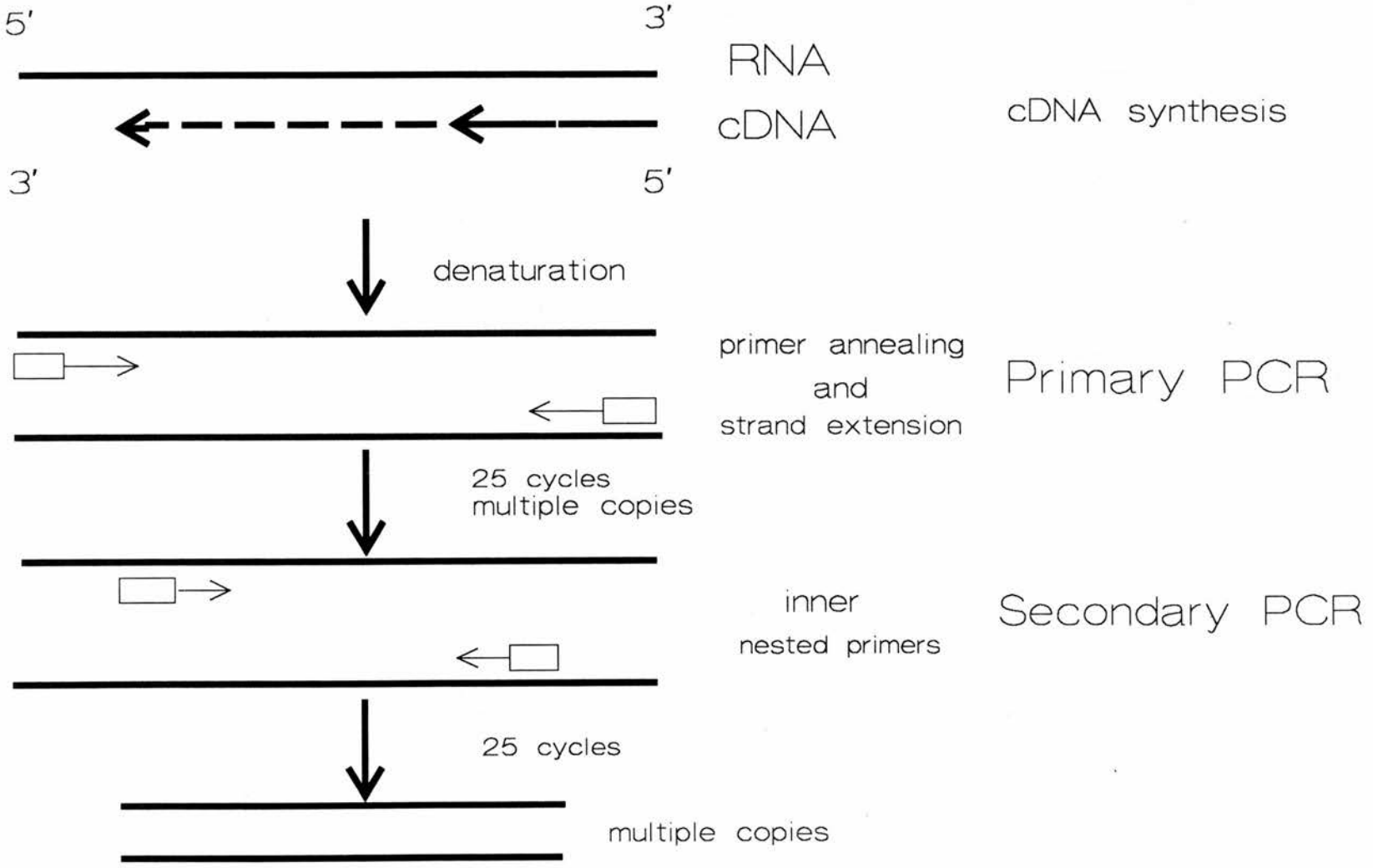
A solution of TNE buffer+0.5% (0.11 M NaCl; 55mM Tris pH 8.0; 1.1mM EDTA pH8.0; 0.55% SDS) plus 1mg/ml proteinase K and 40µg/ml poly-adenylic acid (Poly-A) was prepared prior to use and incubated at 37°C for 10 minutes to inactivate any endogenous RNAses. Serum was either added directly to 400µl of this solution or used to resuspend the pellet, the tubes were mixed thoroughly using the vortimixer. Samples were incubated at 37°C for 2 hours. Extraction was carried out by addition of 450µl of phenol the tubes were mixed thoroughly and centrifuge for 10 minutes at 18000 X g. The aqueous phase was removed and extracted into 450µl of phenol:chloroform:isoamylalcohol (1:1:0.01) containing 75µl of TNE buffer+0.1% (0.1M NaCl; 50mM Tris pH8.0; 1mMEDTA pH8.0; 0.1% SDS), mixing and centrifugation was carried out as described and the aqueous phase removed into 450µl of chloroform:isoamylalcohol (50:1). The aqueous phases was then removed into an equal volume of 100% ethanol and 40µl of 3M Sodium Acetate pH5.2 added. The solution was mixed and nucleic acids precipitated overnight at -20°C. Nucleic acid was pelleted by centrifugation at 18000 X g for 15minutes, the pellet washed once with 80% ethanol and dried prior to resuspending in 25µl of DEPC treated distilled water.

2.5. REVERSE TRANSCRIPTION OF VIRAL RNA

For detection by PCR the RNA must first be reverse transcribed into DNA as the PCR method is ultimately dependent on primer-initiated DNA synthesis, fig 2.1. That is, the DNA replicating enzyme requires a short section of double-stranded DNA on a single stranded template to initiate DNA copying. The efficiency of detection of RNA is critically dependent on the spacing of the primers used in the subsequent PCR (Zhang et al., 1991). The reverse transcripts have a size distribution in which only a minority exceed 1000 bases in length, thus the wider the spacing of primers, the fewer transcripts will be long enough to be amplifiable in the PCR. An efficiency of 5% in the RT reaction with primer spacings of 480bp has been reported, while amplification of cDNA using primer pairs separated by 858bp occurs with an efficiency of 1.8% (pers.comm. Dr.L.Q.Zhang, now at Aaron Diamond AIDS Research Centre, New York). In the work carried out a virus-specific primer for initiation of cDNA synthesis from the viral template was used. Other groups have used random priming with hexameric oligonucleotides (Garson et al., 1990a) however there has been no published data comparing the two methods.

Routinely, 5µl volumes of solubilised RNA were incubated in a reverse transcriptase reaction buffer containing: 50mM Tris-HCl pH8.0; 5mM $MgCl_2$; 5mM DTT; 50mM KCl; 0.05µg/µl BSA; 600µM of each dGTP, dATP, dCTP, dTTP; 20% DMSO; 1.5µM outer anti-sense primer; 10 units RNAsin [Promega] and 10 units reverse-transcriptase (from avian myeloblastosis virus) [Promega] in a final volume of 20µl.

Figure 2.1. REVERSE TRANSCRIPTION AND NESTED PCR



The reaction tubes were incubated at 42^{0}C for 30 min, the primary PCR was set up immediately after the reverse transcription reaction as cDNA produced in this reaction is slightly unstable and long term storage leads to significant reductions in the amount of target DNA amplifiable by the PCR.

2.6. AMPLIFICATION OF DNA BY PCR

The polymerase chain reaction (PCR) is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridise to opposite strands and flank the region of interest in the target DNA. Repetitive cycles of template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase results in the near exponential accumulation of a specific fragment whose termini are defined by the 5′ ends of the primers (fig 2.1). Because the products of one cycle can serve as a template in the next, the number of target DNA copies approximately doubles every cycle. The sensitivity and specificity of the PCR can be enhanced by using a "nested PCR". Following completion of the first PCR reaction a 1µl aliquot of the product is transferred to a second reaction tube containing the PCR reaction mixture with sense and anti-sense primers that lie within the region defined by the outer primers. This highly sensitive technique results in a 10000-fold increase in amplification over that achieved by a single step PCR and has been used to amplify a single copy of the target sequence (Simmonds et al., 1990a). Quantification of viral DNA and RNA can therefore be carried out directly from plasma samples.

The first PCR was set up using 5µl of DNA or cDNA in a final reaction volume of 50µl of 1 X PCR buffer: 10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, 1mg/ml gelatine, pH8.3; containing 33µM each of dGTP, dATP, dCTP and dTTP; 0.5µM of each of the outer nested primers and 20 units/ml of Taq polymerase [Boehringer Mannheim]. Twenty-five heat cycles were used, each consisting of 0.6 min at 94^0C, 0.7 min at 50^0C and 3.0 min at 68^0C. One microlitre of the product was then transferred to a second tube, reaction conditions were the same as for the first PCR but with the inner nested primers and a further 25 heat cycles carried out. The reaction volume for the secondary PCR was generally 20µl but when the amplified DNA was to be sequenced a reaction volume of 50µl was made. The products of the second reaction are sufficient to be visualised under UV light on an 2% agarose [low-melting point agarose, IBI] gel stained with ethidium bromide (final concentration 0.5µg/ml). Figure 2.1. outlines the cDNA reaction and PCR reactions, fig 2.2. is an example of HCV extracted, reverse transcribed, amplified by PCR and run on an agarose gel.

Primers used for the amplification HCV, Parvovirus B19, Canine Parvovirus (CPV), HAV and HIV are shown in Tables 2.1.; 2.2.; 2.3.; 2.4.; 2.5. respectively.

Figure 2.2. AGAROSE GEL OF AMPLIFIED HCV RNA

44 blood donor samples prepared as described in sections 2.4, 2.5, 2.6., PCR product run a 2% agarose gel shown below. From left to right: 1st row: 3 positive blood donor samples; 2nd row: 4 positive control, 2 negative control and 3 positive blood donor samples (controls are placed randomly amongst test samples); 3rd row: 3 positive blood donor samples.

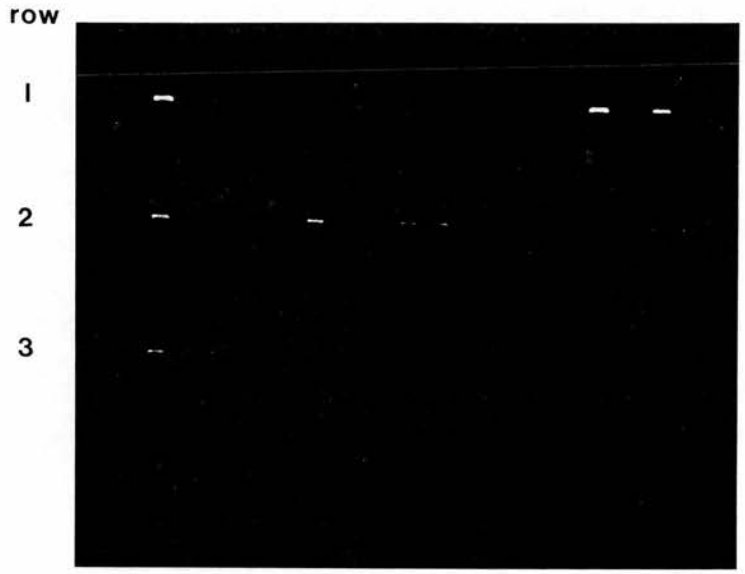


Table 2.1. HEPATITIS C VIRUS PRIMERS

PRIMER/ REGION	LOCATION/ POLARITY	SEQUENCE 5′→3′	POSITION OF 5′BASE	REFERENCE	PRODUCT LENGTH
209/5′NCR	O/AS	ATACTCGAGGTGCACGGTCTACGAGACCT	8	Garson et al. (1990)	289
939/5′NCR	O/S	CTGTGAGGAACTACTGTCTT	297	Okamoto et al.(1990)	
211/5′NCR	I/AS	CACTCTCGAGCACCCTATCAGGCAGT	28	Garson et al. (1990)	251
940/5′NCR	I/S	TTCACGCAGAAAGCGTCTAG	279	Okamoto et al.(1990)	
410/CORE	O/AS	ATGTACCCCATGAGGTCGGC	410	Chan et al.(1992)	356
954/CORE	O/S	ACTGCCTGATAGGGTGCTTGCGAG	54	Simmonds et al.(1993)	
952/CORE	I/AS	TTGCG(G/C/T)GACCT(A/T)CGCCGGGGGTC	353	Simmonds et al.(1993)	332
953/CORE	I/S	AGGTCTCGTAGACCGTGCATCATG	21	Simmonds et al.(1993)	
1204/NS5	O/AS	GGAGGGGCGGAATACCTGGTCATAGCCTCCGTGAA	8309	Not published	406
1203/NS5	O/S	ATGGGGTTCTCGTATGATACCCGCTGCTTTGACTC	7903	Not published	
123/NS5	I/AS	GCTCTCAGGTTCCGCTCGTCCTCC	8250	Simmonds et al.(1993)	315
122/NS5	I/S	CTCAACCGTCACTGAGAGAGACAT	7935	Simmonds et al.(1993)	

Key to Tables : 2.1.,2.2.,2.3.,2.4.,2.5..
O : Outer
I : Inner
S : Sense
AS: Anti-Sense

Table 2.2. PARVOVIRUS PRIMERS

PRIMER	POLARITY	SEQUENCE 5′→ 3′	POSITION OF 5′ BASE	PRODUCT LENGTH
PV1	O/S	GGTAAGAAAAATACACTGT	1390	218
PV2	O/AS	TTGCCCGCCTAAAATGGCTTT	1608	
PV3	I/S	ATGGGCCGCCAAGTACAGGAAA	1415	105
PV4	I/AS	TCATTAAATGGAAAGTTTTCATT	1520	
PV5	O/S	AAGTTTGCCGGAAGTTCCCG	3076	402
PV6	O/AS	AGCATCAGGAGCTATACTTCC	3478	
PV7	I/S	CCCAAGCATGACTTCAG	3118	278
PV8	I/AS	TCTAAATATCTCCATGG	3396	

Table 2.3. CANINE PARVOVIRUS PRIMERS

PRIMER	POLARITY	SEQUENCE 5′→ 3′	POSITION OF 5′ BASE	PRODUCT LENGTH
CPV1	O/S	GGTAAAAGAAATACAGTTCTTTTT	1390	218
CPV2	O/AS	GTTTGTCCAGAACAAATTGCTTT	1608	

Table 2.4. HEPATITIS A VIRUS PRIMERS

PRIMER	POLARITY	SEQUENCE 5′→ 3′	POSITION OF 5′ BASE	PRODUCT LENGTH
HAV1	O/S	AAGTCCATGGTGAGGGGACT	41	231
HAV2	O/AS	CTCCTA/TCAGCTCCATGCTAA	310	
HAV3	I/S	GCCGTTTGCCTAGGCTATAG	73	151
HAV4	I/AS	CGGCCAGAGCCTAGGGCAAG	263	

Table 2.5. HUMAN IMMUNODEFICIENCY VIRUS PRIMERS

PRIMER	POLARITY	SEQUENCE 5′→ 3′	POSITION OF 5′ BASE	PRODUCT LENGTH
HIV1	O/S	TCAGGAGGGGACCCAGAAATT	7316	498
HIV2	O/AS	CCATAGTGCTTCCTGCTGCT	7814	
HIV3	I/S	GGGGAATTTTTCTACTGTAAT	7360	305
HIV4	I/AS	CTTCTCCAATTGTCCCTCATA	7665	

2.7. QUALITY CONTROL OF THE PCR

The Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB) are actively involved in standardisation of HCV-RNA detection. In 1992 a series of samples were supplied to laboratories throughout Europe, called the Eurohep panel. HCV RNA detection was assessed comparing the sensitivity and specificity of the different laboratories. A further HCV Eurohep study is currently being carried out (1993-1994). The author has performed the RT-PCR tests required for both of these studies. The results of the 1992 Eurohep study are presented and discussed in chapter 7, section 7.3..

On a routine basis positive and negative controls were extracted and handled in the same way as test samples. Plasma from a HCV positive haemophiliac was used a positive control. Five serial ten-fold dilutions of the plasma were made with each sample extracted separately. The sample titrated out to the third dilution being positive. This provided an indication of the sensitivity of the extraction procedure on a routine basis. It is important to incorporate such a dilution series as any variation in the positivity cut-off for the control indicates that there may be a problem with the procedure. In particular the enzyme reverse transcriptase is a relatively labile enzyme and batch variation may also occur which could give rise to false negative results being reported for test samples. For every extraction two negative controls were incorporated. At no time did these samples give a positive result.

2.8. DIRECT SEQUENCING OF PCR PRODUCTS

A final PCR volume of 50µl is required to produce sufficient DNA for use in a sequencing reaction. Excess, un-incorporated nucleoside triphosphates and primers must be removed prior to sequencing as these will interfere with subsequent reactions. Initially, samples were purified using a MAGIPREP kit [Promega], DNA was eluted into a final volume of 20µl of 1 X TE buffer (10mM Tris HCl pH7.4; 1mM EDTA pH8.0). In the latter part of the work, single strand sequencing was carried out using DYNABEADS [Dynal Ltd.]. The sample to be sequenced was amplified in a 100µl volume using a biotinylated primer in the secondary PCR. For each sample, 20µl of streptavidin coated beads were washed once with 40µl of 0.1% bovine serum albumin (BSA) pH7.2, followed by washing with 40µl of BW (10mM Tris HCl pH7.5; 1mM EDTA; 2.0M NaCl). Forty microlitres of the biotinylated product was incubated with 40µl of beads resuspended in BW for 15-20 min at room temperature with gentle agitation to enable immobilization. The beads were then washed once with 40µl of BW followed by incubation for 10 min at room temperature with 8µl of 0.15M sodium hydroxide (NaOH) which renders the DNA single stranded. A further wash with 50µl of 0.15M NaOH followed by a wash with 40µl of BW then TE buffer were carried out. The final product was resuspended in 20µl TE buffer pH7.0 and a 5µl aliquote used in the sequencing reaction. The opposite sense primer is used in the sequencing reaction to that which is biotinylated.

The sequencing reaction was carried out following a modification of the Sanger protocol by Winship (Winship, 1989) in which 10% DMSO (tissue culture grade) is

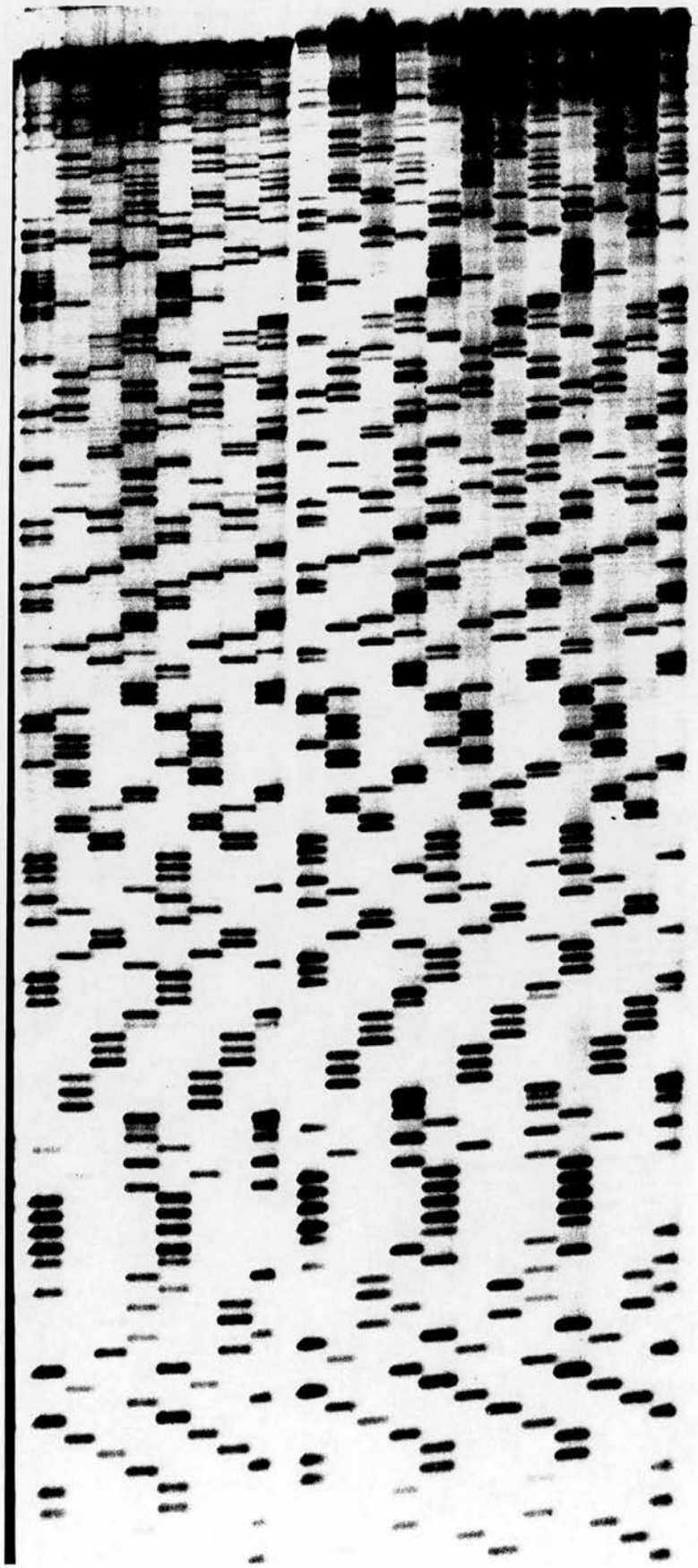
used in the reaction which serves to enhance the intensity of the signal and reduce background. Purified PCR product (5µl) containing approximately 100-200ng of DNA was added to an equal volume of annealing mixture: 10% DMSO; 200mM Tris HCl pH7.5; 100mM $MgCl_2$; 250mM NaCl; 10ng primer). The mixture was boiled for 3 minutes to denature the template. When the DNA was prepared by MAGIPREP, it was immediately placed on ice for ten minutes to minimise template renaturation. DNA prepared by the DYNABEAD method was heated to 65°C for 5min then allowed to cool slowly to room temperature. The template-primer mix was then added to the extension reaction solution: 0.025M DTT; labelling mix (diluted 1 in 20) 7.5µl dGTP, 7.5µl dCTP, 7.5µl dTTP; α-^{35}S-dATP; 2 units of Sequenase enzyme (T7 DNA polymerase). The tubes were manipulated on ice prior to aliquoting into the termination mixture: 80µM dNTP (dGTP, dATP, dCTP, dTTP); 8µM ddNTP (ddGTP, ddATP, ddCTP, ddTTP respectively); 50mM NaCl; 10% DMSO, which was pre-warmed to 37°C. The termination reaction was stopped after 5 min incubation at 37°C by the addition of 4µl of stop solution: 95% formamide; 20mM EDTA; 0.1% bromophenol blue; 0.1% xylene cyanol FF. The samples were heated to 95°C for 2 min prior to loading on a 6% denaturing polyacrylamide gel.

2.8.1. DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

Thoroughly cleaned glass plates were assembled using wedge spacers (0.4mm at the top to 1.2mm at the base) which improve the resolution and spacing of the electrophoresed fragments enabling the reading of a greater number of bases. A 6% gel was prepared by dissolving: 9g of acrylamide [BDH]; 0.45g bis-acrylamide

[BDH]; 75g urea [IBI]; 15ml 10 X TBE (1.34M Tris HCl; 680mM boric acid; 25mM EDTA; pH10.0) in 50ml of distilled water, gentle heat is required to aid dissolution. Polymerisation was initiated by the addition of 0.15g of ammonium persulphate [Sigma] and 20µl of TEMED (N,N,N'N'-Tetramethylethylenediamine) [Sigma] immediately prior to pouring the gel. Denatured samples were loaded onto the pre-run gel (10 minutes at 75 watts) and electrophoresed for approximately 2.5 hours, until the bromophenol blue marker reached the bottom of the gel. The gel was fixed by soaking in 1 litre of 7% acetic acid and 7% methanol for 15 minutes followed by another wash for 15 minutes. The gel was dried under vacuum on a dryer [Model 583 Gel Dryer, Bio-Rad] for 2-3 hours at 80^0C and exposed to X-ray film [Kodak XAR-2] in a light-tight cassette over-night. Films were developed by kind permission of the X-ray department at the Royal Infirmary, Edinburgh. A typical sequencing gel is shown in fig 2.3.

Figure 2.3. SEQUENCING GEL



2.8.2. SEQUENCE ANALYSIS

In phylogenetic studies, the evolutionary relationships among viral strains (or among other groups of organisms) can be illustrated by means of a phylogenetic tree. Dr. Eddie Holmes (now at the Univesity of Cambridge) carried out phylogenetic analysis on sequences obtained in this thesis. Briefly, sequence data can be handled in two different ways: (1) a statistical approach and (2) a method based on the principle of parsimony (parsimony, invariants/evolutionary parsimony). Statistical approaches are widely used and are usually either Distance based methods (for example Fitch-Margoliash or Neighbour-joining) or the Maximum-Likelihood method. Both models make a prediction on how the sequences have changed during evolution. Assumptions are made by all approaches and are as follows:

(a) No variation in nucleotide frequencies during evolution (assumed in all methods).

(b) No between-lineages variation in the rate of evolution.

(c) No between-site variation in rate of evolution (assumed in most methods). Maximum Likelihood methodology allows for some rate variation along the sequence with different classes of site having different rates.

(d) Sites are independent (assumed in most methods). The Maximum Likelihood method allows for 'patches' of correlated sites.

The Maximum-Likelihood model is used in analysis of data in this thesis and relies on the following criteria:

(1) a model (M) (the Felsenstein model of molecular evolution)

(2) the data (D)

(3) the molecular sequence under comparison

(4) the competing hypotheses (H) - always assumed (ie: the alternative tree patterns with associated time of divergence).

The likelihood theory states that hypotheses be judged on the basis of their likelihood. If P(D/H) is the probability of obtaining the data D given the hypothesis H, then the likelihood of an hypothesis H, given the experimentally determined data D, on the given model, is: $L_D(H) = P(D/H)$. In effect, likelihood assesses the probability of the data given the model under the various hypotheses, it does not assess the likelihood of the model, however two competing hypotheses can be taken into account by using the likelihood ratio: $L_D(H1)/L_D(H2)$. The statistical method of likelihood is not the critical element in the estimation of phylogeny, it is the particular model of molecular evolution that is the biological consideration and it is the model that can be altered if necessary.

Nucleotide sequences obtained were compiled by version 2.0 of the programs of Staden (1984) and analysed by programs available in the University of Wisconsin Genetics Computer Group sequence analysis package, version 7.0 (Devereux et al., 1984). Sequences were aligned using PILEUP and LINEUP programs and phylogenetic analysis performed using two different programs available in the PHYLIP package of Felsenstein (version 3.4). A matrix of nucleotide sequence distances were estimated using the program DNADIST, allowing for the calculation of average sequence diversities both within and between samples. Phylogenetic trees were then constructed using the program NEIGHBOR which clusters the matrix of nucleotide distances created by DNADIST. The second PHYLIP program, DNAML

finds the tree of the highest likelihood (the maximum likelihood tree) given a particular stochastic model of molecular evolution and is probably the most reliable.

2.9. QUANTITATION BY LIMITING DILUTION

The sensitivity of the nested PCR for single molecules of target DNA permits a straightforward method of quantitation of viral sequences. Samples were titrated to an end-point in serial ten-fold dilution steps, and the final concentration producing a visible signal can be considered to contain at least one molecule of target sequence. The accuracy of the titration was increased by increasing the number of replicates at each dilution. In practice, the cut-off point was established from ten-fold dilutions then five replicates were set-up at and around this cut-off value. As the distribution of very dilute DNA between samples by macroscopic pipetting is a completely random process, the Poisson formula can be used to calculate the likelihood of positive samples having originally contained one or more molecules of target DNA or RNA. The observed frequencies were converted into molecular concentrations of target DNA by the Poisson formula, $-(\ln f_o)$, where f_o is the frequency of negative reactions (Simmonds et al., 1990a). Levels of RNA in a sample can be determined by titration of cDNA prepared from a suitable concentration of viral RNA, however as described in section 2.3. the efficiency of the reverse transcriptase reaction has to be taken into account when making calculations of viral copy numbers (Zhang et al., 1991). Comparisons made of the positivity rate over a range of concentrations of RNA and the equivalent amount of cDNA showed that unless there was large amounts of product DNA (>100ng) then the result obtained by titrating the RNA or cDNA was equivalent. In practice it is more economical and convenient to titrate the cDNA.

2.10. HCV TYPING

2.10.1. SAMPLE PREPARATION

Following identification of a positive sample by the standard PCR procedure described, the appropriate primary PCR product was retrieved from storage and DNA was amplified in a second round PCR as before but with the modification of supplementing the PCR buffer with 2μCi of [^{35}S]-dATP (Amersham International, Amersham, UK) and reducing the concentration of unlabelled nucleotide triphosphates to 8.25μM. One microlitre of the product was digested with 10 units of the appropriate enzyme. A total reaction volume of 30μl was used in the appropriate restriction buffer supplied by the manufacturer, incubation was carried out at 37^{0}C for 18 hours (overnight). The cleaved product was heated to 65^{0}C for 5 min and 6μl of loading buffer (2% sucrose; 0.1% bromophenol blue; 0.1% xylene cyanol FF) added and mixed prior to loading. The digest products were electrophoresed on a 12% polyacrylamide gel in 1 X TBE at 50 Volts for 18 hours on the Protean II electrophoresis apparatus (Biorad). The gels were fixed in two changes of 2% acetic acid, 10% methanol and 0.3% glycerol (BDH) for 30 min each, and dried for 3 hours then exposed to X-ray film for 3 days prior to development. Figure 2.4. shows a typical RFLP gel prepared by this method.

Figure 2.4a. POLYACRYLAMIDE RFLP GEL : HaeIII/RsaI DIGEST

Migration distances and sizes (in bp) of DNA M_r markers (HaeIII-digested pBR322 DNA) stained separately by ethidium bromide are shown in M. Inferred sizes of bands for the samples are shown on the right (in bp). Different electropherotypes obtained identified by letters along the top (see section 4.3.2. fig.4.1a.).

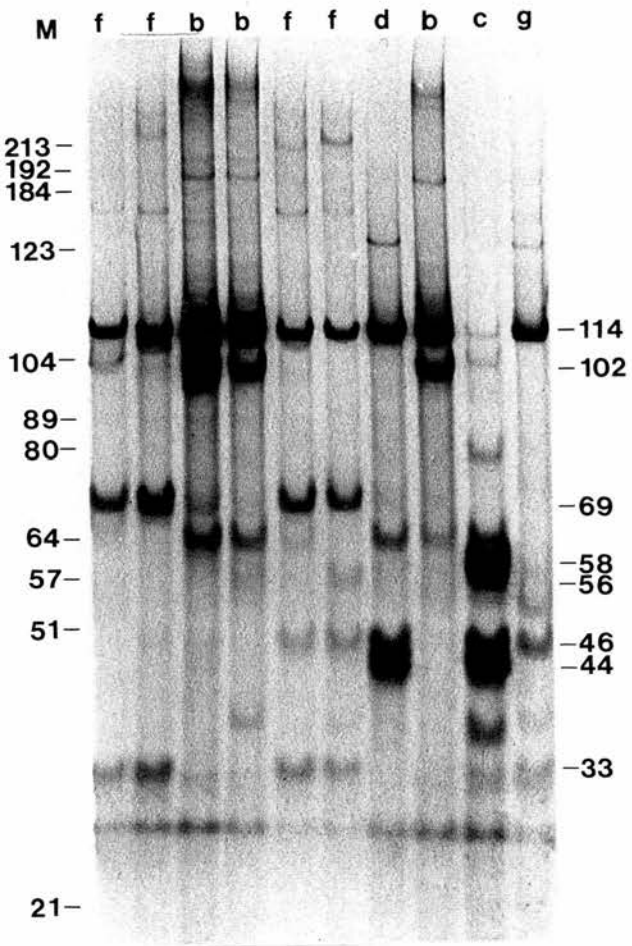
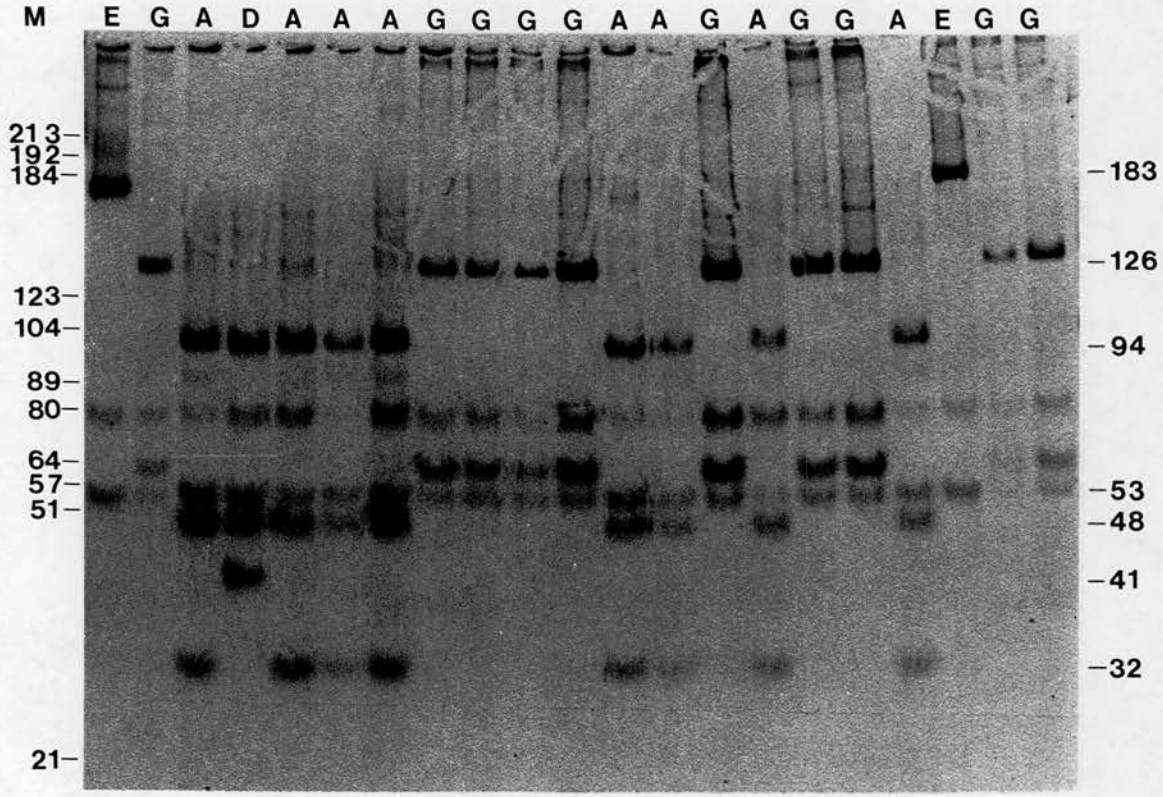


Figure 2.4b. POLYACRYLAMIDE RFLP GEL : ScrFI DIGEST

Migration distances and sizes (in bp) of DNA M_r markers (HaeIII-digested pBR322 DNA) stained separately by ethidium bromide are shown in M. Inferred sizes of bands for the samples are shown on the right (in bp). Different electropherotypes obtained identified by letters along the top (see section 4.3.2. fig.4.1b.).



2.10.2. MOLECULAR WEIGHT MARKERS

There were no commercially available standard DNA size markers in the range of fragment sizes that were obtained. A method that produces 5' end-labelled DNA markers prepared using γ-[^{32}P]dATP and polynucleotide kinases is available, however it was inappropriate to use this method as radiolabel would have to have been ordered specifically for this work. Due to the relatively short half-life of ^{32}P it would have been necessary to order amounts of radiolabel most of which would be wasted making the technique too expensive for the number of samples examined. In addition, the laboratory area where the gel electrophoresis was carried out was not an area authorised for use of ^{32}P and it is possible that the gel drier used would become contaminated with ^{32}P.

In order to overcome the problems associated with the use of ^{32}P, DNA fragments were sized by comparison with migration distances of standard-size DNA markers. The two outermost tracks of the gel were run with a pBR322 digested with HaeIII (Boehringer Mannheim), after electrophoresis the marker tracks were excised and stained with ethidium bromide and the migration distances measured. A plot of M_r versus relative migration (Rf) was used to estimate the size of the bands on the autoradiograph after exposure and development.

2.10.3. IMPROVED RFLP METHOD

During the course of this work an agarose gel became available that was capable of resolving DNA fragments down to 3 base pairs in size. Modification to the original method was made which enabled a larger number of samples to be analysed in a shorter period of time. Samples to be tested were amplified in a 25µl secondary PCR reaction volume. The whole sample was then digested with the appropriate combination of restriction enzymes as described in section 2.10.1. in a final volume 30µl. The whole sample was then run on a 4% METAPHOR [FMC Ltd] agarose gel stained with ethidium bromide. Figures 2.5a and b. show typical RFLP agarose gels. This method enabled a standard set markers to be run at the same time.

Figure 2.5a. AGAROSE RFLP GEL : HaeIII/RsaI DIGEST

Sizes of bands (bp) shown on right. Different electropherotypes obtained labelled by letters along the top (see section 4.3.4. fig 4.8a.).

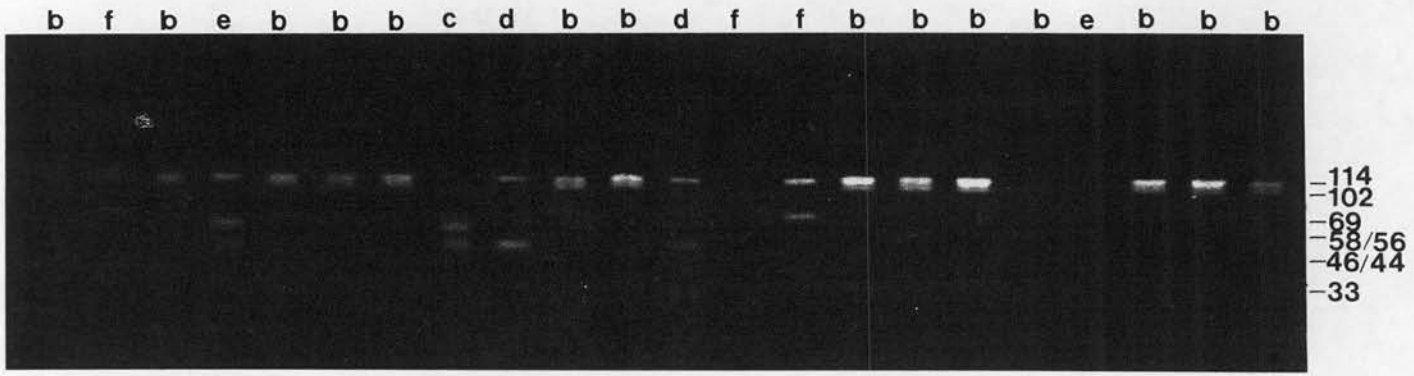
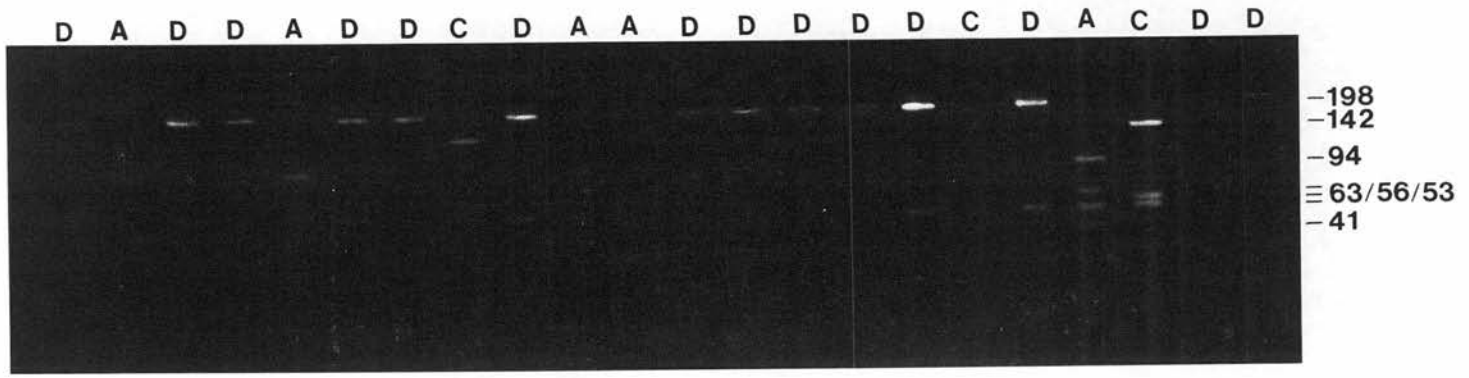


Figure 2.5a. AGAROSE RFLP GEL : MvaI/HinfI DIGEST

Sizes of bands (bp) shown on right. Different electropherotypes obtained labelled by letters along the top (see section 4.3.4. fig 4.8c.).



2.11. DIGOXIGENIN LUMINESCENT DETECTION OF NUCLEIC ACIDS

Detection of digoxigenin (DIG)-labelled nucleic acids by enzyme immunoassay with luminescence on nylon membranes has largely replaced detection systems using ^{32}P radiolabelled DNA, although the basic principle remains the same. The hapten DIG is bound by a spacer arm to uridine-nucleotides and incorporated enzymatically into the nucleic acid probe by random primed DNA labelling. Hybridisation and blocking are carried out, followed by detection of DIG-labelled probes using high affinity anti-DIG-antibody Fab-fragments conjugated to alkaline phosphatase (AP). The chemiluminescent substrate AMPPD (3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane) is dephosphorylated by the alkaline phosphatase, the resulting decomposition via an unstable intermediate produces light as a glow which can be detected by X-ray film.

PCR positive samples were run on a 2% agarose gel stained with ethidium bromide (as in section 2.6.). The DNA was denatured by incubating the gel in 100ml of denaturing solution (0.5M NaOH; 1.5M NaCl) with gentle agitation for 1 hour. Denaturing solution was removed and replaced with 300ml of neutralising solution (1.5M NaCl; 0.5M Tris HCl; pH8.0) for a further 45 min, followed by two washes with 2 X SSC (0.3M NaCl; 0.03M Na citrate; pH 7.0). Southern transfer was carried out overnight to a nylon membrane [Boehringer Mannheim] and dried at 80^{0}C for 2hours the following day. The filter was prehybridised in a roller bottle with 30ml of hybridisation solution (50% formamide; 5 X SSC; 2% blocking reagent [supplied in the Boehringer Mannheim DIG Luminescent Detection Kit]; 0.1% N-lauryl

sulphate; 0.02% SDS [sodium-dodecyl sulphate]) for 1 hour at 42°C. Probe DNA was labelled by mixing 100ng of DNA with 3.2µl dNTP mix (kit dCTP:dGTP:dATP); 1.8µl dTTP/dUTP mix (1:1; dTTP[0.5mM]:dig dUTP[0.3mM]); 2.0µl reaction mix (kit); 1µl of Klenow enzyme, in a total volume of 20µl and incubated at 37°C for 1 hour. The labelled probe was then added to the filter in the roller bottle containing the hybridisation solution and incubated at 42°C overnight. The filter was then washed two times with 2 X SSC + 0.1% SDS for 5 min at room temperature, followed by a further two washes with 0.1 X SSC + 0.1% SDS for 5 min at 68°C. Chemiluminescent detection of target was carried out at room temperature by washing the filter in 30ml of washing buffer (0.1M Maleic Acid; 0.15M NaCl; pH7.5) + 0.3% Tween 20 for 5 min. This buffer was removed and replaced with 20ml of washing buffer + 1% blocking agent for 30 min, the anti-digoxigenin-AP, Fab fragments were diluted into this solution at 1:10,000, and incubated for 30 min. Unbound conjugate was removed by washing two times with 20ml washing buffer + 0.3% tween 20 for 15 min. Equilibration was carried out by incubation with 20ml of buffer E (0.1M Tris HCl pH9.5; 0.1M NaCl; 50mM $MgCl_2$). Substrate solution AMPPD was diluted 1:100 in buffer E and incubated with the filter for 5 min. The filter was placed on a backing sheet of Whatman 3MM filter paper, sealed in Saran Wrap and incubated at 37°C for 5 min then exposed to X-ray film for 30 min and developed as section 2.8.1..

CHAPTER 3

3. HEPATITIS C VIRUS : GENERAL INTRODUCTION

3.1 POST-TRANSFUSION NON-A NON-B HEPATITIS

The hepatitis C virus is recognised as the main cause of PT-NANBH worlwide. Until recently the diagnosis of HCV infection was one of exclusion. Individuals presenting to the clinician with symptoms of jaundice, hepatic tenderness, increased serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase were subjected to routine diagnostic tests for hepatitis B virus (HBV), hepatitis A virus (HAV), Epstein-Barr (EBV) and cytomegalovirus (CMV). In those cases where infection was proven not to be caused by any of these agents the diagnosis of non-A non-B hepatitis (NANBH) was made. Until recently the NANBH agent was responsible for 60 to 90% of cases of post-transfusion hepatitis (PTH) (Prince et al., 1974; Tabor et al., 1978; Alter et al.,1975). The incidence of PTH-NANBH in recipients of blood was reported to be very low in the UK at 0.26% (Contreras et al., 1991) and between 2 and 4% in Northern Europe, 6 and 18% for Southern Europe and between 10 and 12% in the United States in 1991 (van der Poel 1991a).

3.2. DISCOVERY OF THE HEPATITIS C VIRUS

For many years the agent responsible for NANBH remained elusive. Initial characterisation and physical identification of the virus was made by using the chimpanzee as an infectivity model. Sera proven to induce liver disease in chimpanzees could be treated in different ways and the effect on subsequent transmission measured. In this way it was shown that the agent was inactivated by chloroform and the deduction made that the virus contained lipid and was likely to

be associated with the surface membrane. Filtration studies showed that the virus was approximately 30 to 60 nm in diameter. With this information it was possible to deduce that the NANBH agent was a small, lipid-enveloped virus possibly related to the class of small arthropod-borne RNA viruses, and was provisionally classified with the togaviridae (Bradley et al., 1985).

Identification of the NANBH agent proved difficult since conventional immunological methods failed to identify specific antibodies or virus antigens. This failure could have been due to a lack of virus-specific antibody or to insufficient production of virus antigens in NANBH infections. A novel approach was taken by Choo et al. (1989) in which the infectious agent was cloned without prior characterisation. Large quantities of a highly infectious chimpanzee plasma were subjected to ultracentrifugation in order to concentrate the virus. Nucleic acid was extracted from the pellets and denatured, as the nature of the genome was unknown, synthesis of cDNA from both RNA and DNA using random hexamers was attempted. A cDNA library was constructed in the bacteriophage λgt11, a vector which allows the efficient expression of cDNA-encoded polypeptides. This library was then screened for expression of virus antigens using serum from a patient with chronic NANBH. Screening of approximately one million clones led to the identification of one positive cDNA clone, termed 5-1-1. The 155 base-pair insert in this clone was then purified and used as a hybridisation probe on the same library and a larger overlapping clone (353 base-pairs) was identified. The cloned DNAs did not hybridise with human or chimpanzee DNA, however one of the strands was

homologous with a 5-10 kb nucleotide single-stranded RNA. Binding to oligo(dT)-cellulose occurred indicating the presence of either a 3′ terminal polyadenylated sequence or an A-rich tract within the molecule. Sequence analysis of this and two other overlapping clones indicated that the immunoreactive peptide possessed one continuous, translational open reading frame (ORF). In order to express the ORF in a system that could generate large quantities of the resulting polypeptide, the DNA sequence was inserted into a plasmid containing the human superoxide dismutase gene. When expressed in bacteria the resultant fusion protein reacted on immunoblotting with serum from 7 of 11 patients with NANBH, and none of 10 control sera from individuals with non-NANB induced hepatitis. In addition, seroconversion was demonstrated in 4 chimpanzees experimentally inoculated with the NANBH agent, but not in 7 animals infected with HAV or HBV.

This work led to the further characterisation of the agent of NANBH, now termed hepatitis C virus (HCV), a positive stranded RNA genome of at least 10,000 nucleotides (Choo et al., 1989).

3.3. GENOME ORGANISATION AND FUNCTION

Following the identification and characterisation of the hepatitis C virus by Choo et al.(1989;1991) further reports confirmed that HCV exists as a positive-stranded RNA genome containing a large open reading frame encoding 3010 to 3033 amino acids (Kato et al.,1990; Takamizawa et al.,1991; Okamoto et al.,1991). The ORF is flanked by a 5′ non-coding region (5′NCR) of 341 nucleotide in length that is important in

translation initiation (Yoo et al.,1992), and a short 3′ NCR (Han et al.,1991) (fig.3.1.). Eukaryotic mRNAs are translated by a mechanism known as ribosome scanning. The 40S ribosomal subunit binds adjacent to the 5′ end of the mRNA, mediated by interaction between a 5′methylated cap structure and a cellular initiation factor complex. HCV appears to have a mechanism for translation initiation that enables cap-independant translation. Although the exact mechanism is yet to be defined it is thought that translation is initiated from an AUG codon that is located at an internal site from the 5′NCR (an internal ribosome entry site, IRES) (Tsukiyama-Kohara et al.,1992). Several AUG codons are located upstream of the translation initiation site, however it is unlikely that these function as alternative start sites. If IRES initiated translation does occur, as is the case for picornaviruses, the virus may gain a major selective advantage since viral infection could effectively inactivate the cellular cap-binding protein by production of an inhibitory protein which leads to the arrest of cellular protein synthesis. Computer analysis of the 5′NCR has shown that sequences form secondary structures indicative of a stable stem-loop formation which may serve as a ribosome landing pad. *In vitro* studies have shown that sequences immediately upstream of the initiator AUG codon play a key role in efficient translation initiation (Wang et al.,1993).

Cloning of the HCV genome has enabled a proposed classification, however the identification of the proteins derived from translation of the ORF and subsequent processing until recently could only be inferred from related viruses. Identification of HCV proteins has been difficult as a cell culture model did not exist that allowed

Figure 3.1. ORGANISATION OF THE HCV GENOME

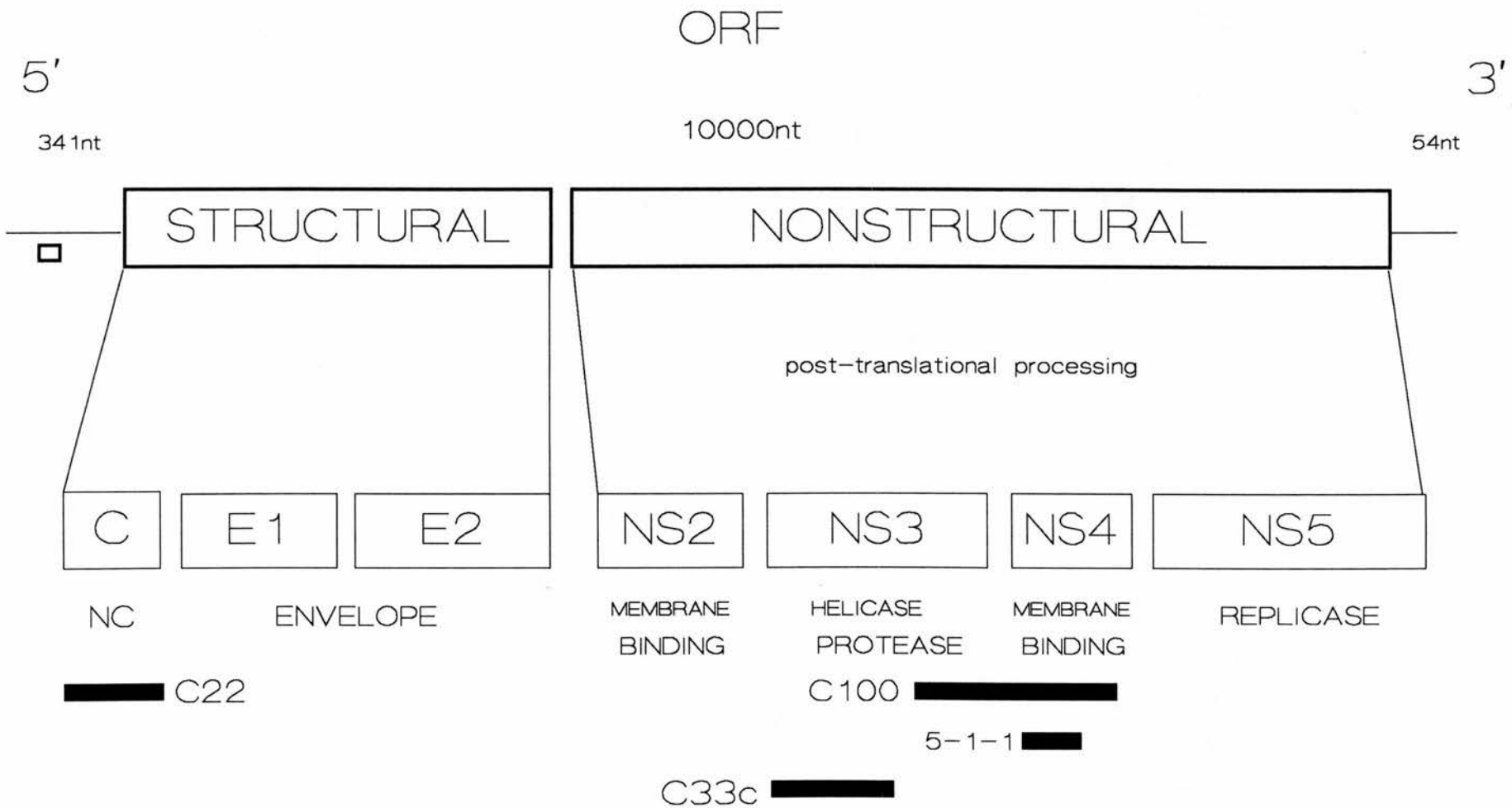


Figure 3.1. LEGEND

Schematic representation of the HCV genome and regions encoding the structural and non-structural proteins. The top line depicts the viral genome, 5′ and 3′ non-coding regions are indicated as single lines (secondary structure not shown). □ indicates the existence of an internal ribosome entry site (IRES) within the 5′NCR proximal to the initiator codon.

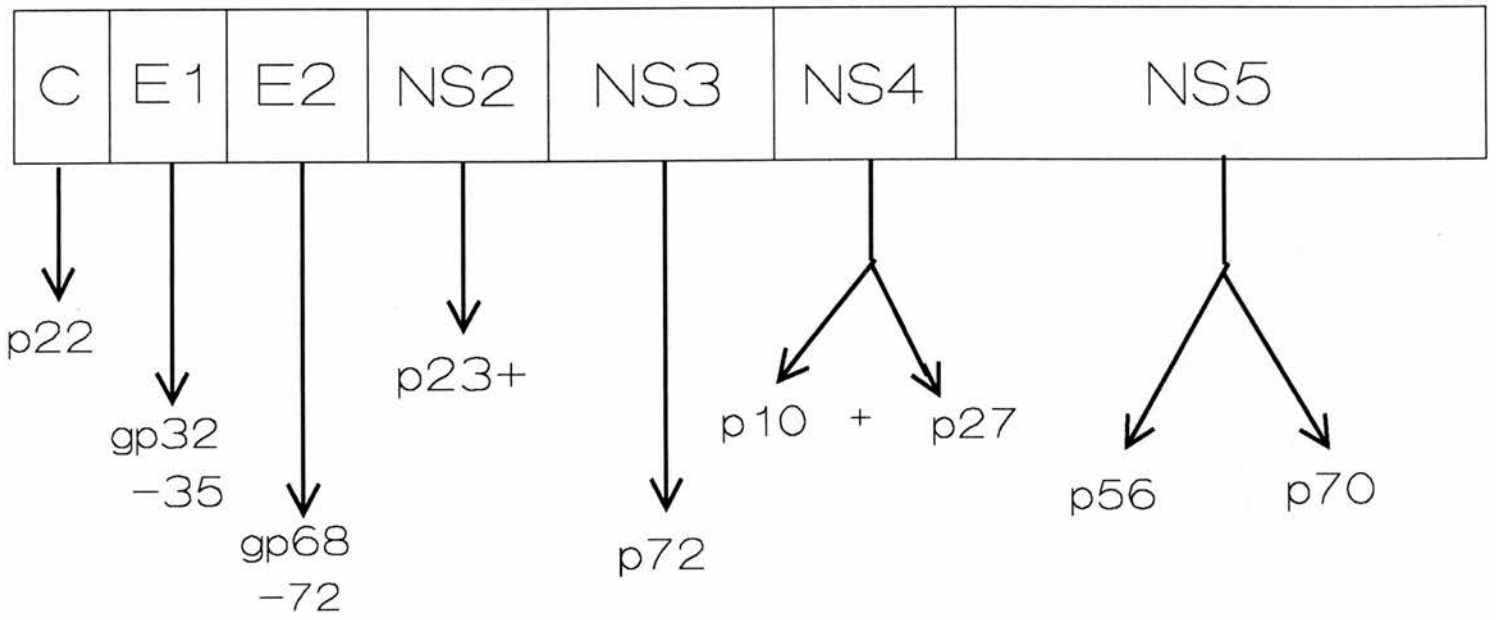
The processed proteins are shown on the second line, there is evidence for additional processing of NS4 and NS5. NC indicates the nucleocapsid.

The proposed function of the proteins is indicated as well as the location of the recombinant antigens used in anti-HCV antibody assays.

in vitro studies on HCV to be done, expression of the full-length recombinant genome in mammalian cells was required before particular domains of the ORF could be accurately assigned to processed proteins. Using a vaccinia virus infection and transfection system along with HCV antibody-positive human sera and monospecific antibodies, the proteins encoded by the putative structural and non-structural regions of the ORF of HCV were identified as core (p22), E1 (gp32-35), E2 (gp68-72), NS2 (p23), NS3 (p72), NS4a and b (p10 and 27 respectively) and NS5a and b (p56 and p70 respectively) (Selby et al.,1993) (fig 3.1. and 3.2.). Expression of the N-terminal putative structural proteins *in vitro* and *in vivo* also enabled the identification of core and envelope glycoproteins (Harada et al.,1991; Hijikata et al.,1991a; Kumar et al.,1992; Matsuura et al.,1992), however it remains to be determined whether co-expression of the non-structural proteins influences processing of the structural proteins. The NS3 region encodes a protein of dual function, it has a potential catalytic triad similar to that of trypsin-like serine proteases and contains a consensus helicase domain thought to be involved in template replication and possibly translation. The NS5 region is thought to correspond to the replicase protein as it contains a domain highly conserved amongst all viral RNA-dependent RNA polymerases (Choo et al., 1991). Figure 3.2 indicates the cleavage points of the HCV polyprotein. The function of the NS2 and NS4 proteins have yet to be determined. Identification and immunostaining of the proteins encoded by the HCV genome suggests that most of them are present in the ER, and only NS3 and NS5a may be soluble and therefore have a cytoplasmic location.

Figure 3.2. CLEAVAGE POINTS OF THE HCV POLYPROTEIN

Schematic representation of the processed proteins encoded by the HCV genome. The proteins derived from the HCV ORF and their molecular weight are shown. The split arrows denote additional processing of NS4 and NS5. Envelope glycoproteins are marked gp. The + after gp23 signifies another possible cleavage product.



3.4. CLASSIFICATION OF HEPATITIS C VIRUS

As yet HCV has not been assigned to a particular virus group. In order to understand the rationale behind the proposal to assign HCV to a separate genus within the Flaviviridae, it is necessary to outline the basis of the classification system.

3.4.1. TAXONOMY OF RNA VIRUSES

Until recently virus groupings have been based on a variety of characteristics such as the structure of the virion; the host range (especially for plants and animals); the transmitting vector; the disease syndrome and antigenic cross-reaction (for closely related viruses). A refinement to this method is a classification based on the physical structure of virus particles, and their stability in the presence of various chemicals and physical agents. A recent classification scheme recognises families of viruses and " super-groups " of families, which are thought to reflect ancestral relationships among otherwise divergent viruses. The criteria for membership of a super-group is mainly based on similarities in genome organisation and structure, for example in the order, number, and type of genes along the genome, the nature of modifications at the 3′ and 5′ ends of the RNA, and the RNA polarity (ie: (+) or (-) strand viruses). Positive strand virus genomes serve as a messenger RNA, following entry into the cell, virus RNA binds to ribosomes and is translated in its entirety. The product of this translation, the polyprotein, is then cleaved into proteins the main function of which is to serve as structural components of the virion. The (+) strand also serves as a template for synthesis of complementary (-) strand RNA by a polymerase derived from cleavage of the polyprotein. Negative strand RNA then serves as a

template to make more (+) strands. Progeny (+) strands then serve as mRNA templates to make more (-) strands and constituents of progeny virus particles. The genome of (-) strand RNA viruses function alternatively as templates for transcription and replication. The virus genome must be transcribed to make mRNA, since the cell lacks the appropriate enzymes all (-) strand viruses package in the virion an RNA dependent RNA polymerase along with the viral genome. Transcription of the virus genome is the first event after entry of the virus into cells. This yields functionally monocistronic mRNAs, (+) strands, each specifying a single protein. Replication begins under the direction of newly synthesised virus proteins, a full-length (+) strand is made and serves as a template for the synthesis of (-) strand genomic RNAs. Other factors include the occurrence of post-translational processing; the production of subgenomic RNAs and readthrough of stop codons.

At present four super families of RNA viruses are recognised:

SUPERFAMILY I : Alphavirus-like families

SUPERFAMILY II : Picornavirus-like families

SUPERFAMILY III : Negative stranded viruses

SUPERFAMILY IV : Double stranded viruses

Sequence data has shown that there are regions of amino acid sequence similarity in the replicase proteins of certain groups of viruses of plants and animals (Alquist et al., 1985; Franssen et al., 1984; Haseloff et al., 1984), suggesting that the different superfamilies have each descended from a common ancestor. On the basis of

sequence similarities and genome order it is proposed that all positive-stranded RNA viruses, ie: those that contain the messenger sense RNA as their genome have evolved from a common ancestor. Members of these superfamilies include viruses that infect many different hosts (eg: plants, insects and higher animals) and have a variety of divergent morphologies.

3.4.2. RELATIONSHIP OF HCV TO OTHER VIRUSES

The structure and organisation of the HCV genome shows similarities to other positive-stranded RNA viruses and in particular, based on genome organisation and hydrophobicity plots, it has been suggested that HCV be classified in the family Flaviviridae (Choo et al., 1991; Han et al., 1991). The Flaviviruses were previously grouped within the Togaviridae (which includes the genera Alphavirus and Rubivirus), but have recently been classified as a single genus of the family Flaviviridae. Currently there are sixty-eight members of the Flaviviridae which can be divided into three different groups based on their mode of transmission; fifty are transmitted by mosquitos, eleven by ticks with the remainder having an unknown vector. The mosquito borne members can be divided into several sub-groups based on serological cross reaction. The Flavivirus virions contain a nucleocapsid of 25-30 nm diameter, surrounded by a lipid bilayer derived from the host cell membranes containing the envelope proteins E and M. The diameter of the virion is approximately 40nm with surface projections of 5-10 nm. The genomes of Yellow Fever Virus, West Nile Virus, Murray Valley Virus, St. Louis Encephalitis, Kunjin, Japanese encephalitis, Tickborne encephalitis and Dengue serotypes 1, 2 and 4 are

single stranded RNA of positive polarity and approximately 11 kilobases in length. The RNA has a cap at the 5′ terminus (m7GpppAmp) (Chambers et al.,1990), in common with the genomes of negative strand viruses and reoviruses it lacks a 3′ terminal poly (A) stretch. The RNA forms a stable hydrogen-bonded stem-and-loop structure at the 3′ terminus (similar to a plant-virus 3′ termini). Short self-complementary nucleotide sequences at the extreme 5′ and 3′ termini of the genome RNA are observed (similar to that found in negative strand viruses). The Flavivirus genome contains a single long open reading frame (ORF) of over 10000 bases, translated as one polyprotein, from which virus proteins are produced by post-translational cleavage (fig.3.3.). Structural proteins are found at the 5′ end and the non-structural components including the RNA polymerase at the 3′ end, a similar arrangement to that found in picornaviruses. Several regions of the genome are conserved in sequence and structure, ninety nucleotides at the 3′ terminus form a stable hairpinloop with the 3′ terminal dinucleotide being hydrogen bonded making it inaccessible to end labelling. Short stretches of conserved and repeated regions in the 3′ non-coding region are found, one stretch of eight contiguous nucleotides in the 3′ untranslated region is complementary to eight nucleotides at the 5′ end (although there is no extensive sequence conservation at the 5′ termini). These conserved sequences are possibly important in cyclisation of the RNA in a similar manner as alphaviruses and bunyaviruses undergo cyclisation which may be important in replication (although there is no direct evidence in flaviviruses).

Figure 3.3. ORGANISATION OF THE FLAVIVIRUS GENOME

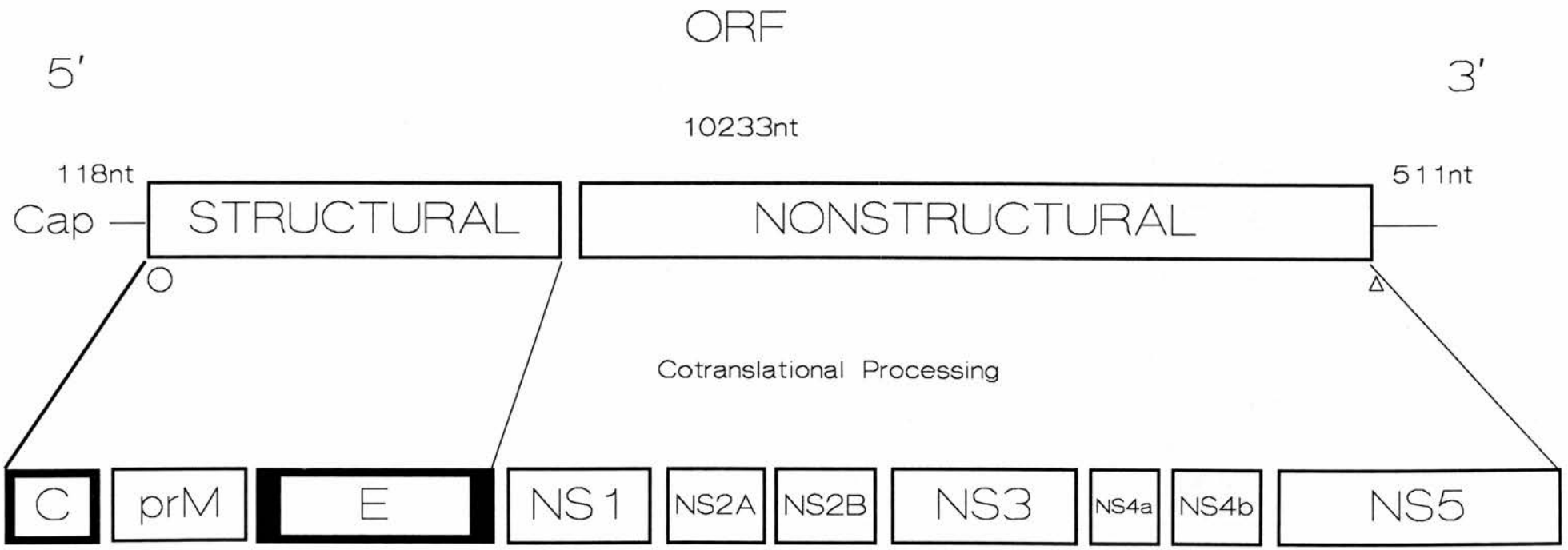


Figure 3.3. LEGEND

Schematic diagram of the flavivirus genome and polyprotein processing. The top line depicts the viral genome, the structural and nonstructural protein coding regions, the 5′ cap, the 3′ end. The 5′ and 3′ untranslated regions are indicated as single lines. Boxes below the genome indicate precursors and mature proteins generated by the proteolytic processing cascade, solid boxes indicate structural proteins while open boxes are nonstructural proteins. ◯ represents the translation initiating AUG; ▵ is the UGA termination codon.

3.4.3. SIMILARITIES TO OTHER POSITIVE STRAND VIRUSES

Virus specified proteinases are common to many positive strand virus families as well as sequence characteristics of NTP-binding domains, helicase enzymes and RNA dependent RNA polymerase activity. Flaviviruses were originally grouped with alphaviruses based on similarities in morphology and their mode of transmission. Genome organisation and expression of the flaviviruses indicate that a similar strategy to picorna-like viruses is used. Both virus genomes encode a single polyprotein that begins with the structural proteins and is processed by proteolytic cleavage to yield the mature viral proteins. In contrast, virion morphogenesis and structural protein processing of flaviviruses are very similar to rubella virus (Frey and Marr 1988), which make up a separate genus in the togaviridae family along with the alphaviruses (see section 3.4.1.). A membrane anchored precursor form of the C protein is probably utilised by both viruses and envelopes are acquired by budding through intracellular membranes. The pestiviruses are probably the most closely related to the flaviviruses, genome sequences for bovine viral diarrhoea virus (BVDV) and hog cholera virus (HoCV) suggest a similar genome arrangement and replication strategy to flaviviruses (see section 3.4.4.). Homology between the flaviviruses and the pestiviruses is mainly confined to certain motifs in the NS3 and NS5 domains with little amino acid sequence homology seen elsewhere. (Figure 3.4. shows the aligned sequences of flavivirus NS3 proteins and cellular proteases with HCV added as a comparison).

Figure 3.4.

The NS3 protease domain. Aligned sequences of flavivirus NS3 proteins and cellular proteases surrounding the putative catalytic triad (shown by the bold type) of the serine protease domain. Also shown are sequences from two pestiviruses (numbered from the beginning of the ORF), bovine viral diarrhoea virus (BVDV) and hog cholera virus (HoCV). Hepatitis C virus (HCV) is included for comparison (see section 3.4.3.).

```
YF     (45)     GGVFHTMW**H**VTRG [13aa] WASVKE**D**LVAYGGSW [44aa] ALDYPSGT**S**GSPIVN RNG EVIGLYGNGI
BVDV   (1740)   QGGISSVD**H**VTAG [22aa] EYGVKT**D**SGCPDGAR [40aa] DLKNLKGW**S**GLPIFEASSG RVVGRVKVGK
HoCV   (1650)   QGGISSVD**H**VTCG [26aa] EYGVKT**D**SGCPEGAR [40aa] DLKNLKGW**S**GLPIFEASSG RVVGRVKVGK
HCV    (342)    NGVCWTVY**H**GAGT [13aa] YTNVDN**D**LVGWPAPQ [41aa] PISYLKGS**S**GGPLLCP AG HAVGIFRAAV
```

3.4.4. COMPARISON OF THE PESTIVIRUS BOVINE VIRAL DIARRHOEA VIRUS WITH MEMBERS OF THE FLAVIVIRIDAE

Table 3.1. highlights some of the main differences and similarities between pestiviruses, rubiviruses, flaviviruses and hepatitis C virus. Nucleotide sequence comparisons of BVDV with the three sub-groups of flaviviruses show that there are no extensive regions of sequence similarity although several small tracts of 17 to 30 nucleotides are similar in BVDV and YFV. More information is gained from comparisons of the genetic organisation and detailed information from the protein coding regions. Hydropathic profiles of BVDV and YFV show striking similarities and also the organisation of BVDV protein coding domains along the ORF is paralleled by YFV. The similarity in hydropathic profiles in the absence of nucleotide homology is important. The several short similarly aligned regions of highly homologous amino acids in two NS proteins may indicate that the representative proteins share similar functions for which the conserved sequences may be essential. The available data is sufficient to distinguish BVDV from members of the Togaviridae however it still remains to be defined if it is more appropriate to classify BVDV and pestiviruses as part of the family Flaviviridae. Definition of the nature of the actual virion structural polypeptides of BVDV would assist in drawing analogies with the flaviviruses (Collet et al., 1988).

Table 3.1.

	PESTIVIRUS	RUBIVIRUS	FLAVIVIRUS	
	BVDV	RUBELLA	YFV	HCV
Genome size (nt)	12573	11000	11000	9401
Polarity	positive	positive	positive	positive
RNA	single size	viral genomic (49S) subgenomic (26S)	single size	single size
Poly A tract	not present	present	not present	in some isolates
Single ORF	yes	no	yes	yes
Translational strategy	co and post translational processing	post translational processing	co and post translational processing	post translational processing
Assembly and maturation	at perinuclear ER and golgi membranes	budding at plasma membrane	at perinuclear ER and golgi membranes	unknown
Inactivation by protease	trypsin-sensitive	trypsin-resistant	unknown	unknown

3.4.5. CURRENT CLASSIFICATION OF HCV

As indicated in the introduction to this section 3.4. the current proposal is to assign HCV to a separate genus within the family Flaviviridae. Section 3.4.4. then showed how the pestivirus BVDV is closely related to the flaviviruses. HCV shows a very close relationship to the pestiviruses with significant sequence similarities in the NS-3 and NS-5 proteins, in addition to regions of nucleotide homology in the 5′ NCR. Similarities in the secondary RNA structure adopted by the respective RNAs during internal base-pairing (Brown et al.,1992) are observed. The stem-loop structure within the 5′ NCR of HCV that has been shown to be necessary for internal ribosomal binding or entry (IRES) in HCV (Tsukiyama Kohara et al.,1992) is almost identical in shape to that in the equivalent region of pestiviral RNA genomes (Brown et al.,1992). In contrast, flaviviruses have a short 5′ NCR and a cap-dependent ribosomal scanning mechanism of translation initiation (Chambers et al.,1990). Both pestiviruses and HCV show sequence diversity in the region of the genome encoding the highly glycosylated envelope proteins. Comparison of the gene sequences for the E2/NS-1 protein of HCV (Hijikata et al.,1991a; Weiner et al.,1991; Kato et al.,1992) and the equivalent gp53 proteins of the pestiviruses (BVDV and HoCV) indicate alternating constant and hypervariable regions not observed with flaviviruses. Frequent potential N-linked glycosylation sites are observed in the E1 and E2/NS-1 protein of HCV and gp48, gp25 and gp53 proteins of pestiviruses and it is possible that this contributes to their low buoyant densities on gradient centrifugation (approximately 1.08- 1.11 g/cm^3 for HCV and 1.09-1.16 g/cm^3 for pestiviruses, in

comparison to 1.20 g/cm^3 for flaviviruses (Miyamoto et al.,1992; Takahashi et al.,1992)).

3.5. RNA VIRUS VARIABILITY

3.5.1. RATE OF DIVERGENCE OF RNA VIRUSES

The genomes of RNA viruses diverge very rapidly. The mutation rate is thought to be approximately 10^{-4} per nucleotide per round of replication (Steinhauer and Holland, 1987). This rapidity of evolution may be a consequence of the lack of proofreading activity in the RNA dependent RNA polymerases. The size of an RNA virus genome may be limited by this high mutation rate (Reanney, 1982). The potential and the actual rates of divergence tend to differ. Potentially, the rate of divergence could be very rapid due to the high mutation rate and the large number of rounds of replication of an RNA virus per year. However a limit is imposed on the actual rate of divergence (ie: mutation of residues in the genome) by selection pressures since most mutations are deleterious or incur disadvantages to the parental population. Studies on RNA viruses estimate divergence rates of 0.03 to 2.0 % per year (Smith and Inglis 1987); although this figure probably overestimates the basal rate of divergence, it is significantly higher than the rate of divergence in animals which is 1 % per million years (Britten 1986). The implications of such a high rate of RNA sequence divergence are considered in relation to HCV in the next section.

3.5.2 IMPLICATIONS OF HIGH MUTATION RATES

The degree of variation observed within the HCV genome is comparable to that of other RNA viruses. However HCV, in contrast with most other RNA viruses, is able to establish a chronic infection which results in a progressive disease in many exposed individuals. The relatively high rate of nucleotide mis-incorporation in the replication of the HCV genome may contibute to the generation of novel antigenic variants and therefore provide a mechanism for " immune escape ". This theory is supported by evidence of a high degree of envelope sequence variability in discrete hypervariable regions of the E1 and E2 genes (Kato et al., 1992). Changes in these regions would possibly alter the antigenicity of the virus and allow " escape " from the hosts neutralising antibodies enabling persistent infection. However, there is evidence that some persistently infected individuals show little change in envelope sequence over a period of time (Kumar et al., 1993). In others, antibody levels are detected that are similar or greater in frequency in individuals who are chronic carriers of HCV compared with those who have cleared the virus and become non-viraemic (Chein et al.,1993). These observations would argue against the " immune escape " hypothesis. Microheterogeneity of virus sequences within an infected individual is proposed as the basis of the mechanism of chronic virus infection for HIV as well as HCV. The extent to which the immune system plays a role in the evolution of the envelope proteins and in the prevention of re-infection are important questions that await answer, and are undoubtedly of importance in the quest to develope vaccines.

3.6. HEPATITIS C VIRUS SEQUENCE VARIABILITY

3.6.1. INTRODUCTION

Hepatitis C viruses are heterogeneous, comprising several distinct major "types" (Enomoto et al., 1990; Tsukiyama Kohara et al., 1991) that display in the region of 70% nucleotide sequence homology overall between types. This corresponds approximately to the degree of variability which exists between different serotypes of other flaviviruses (Chambers et al., 1990). Comparison of nucleotide and amino acid sequences of different variants of HCV suggests the existence of distinct sequence groups. Analysis of the complete genome sequences of HCV variants from Japan (HCV-J, Kato et al., 1990 and HCV-BK, Takamizawa et al., 1991) show ninety-two percent nucleotide sequence identity, compared with only 79% between HCV-J and HCV-1 from the USA (Choo et al., 1991). Nucleotide sequence comparisons in the NS-5 region of HCV between a series of isolates from infected individuals in Japan (Enomoto et al.,1990) divides viruses into two main types that differ by 33%, both of which can be further divided into two sub-types each differing by 20%. The sub-divisions of the first group K1 and PT corresponded to the genome sequences HCV-J/HCV-BK and HCV-1 respectively. Complete genome sequences of the other major type (K2) have also been obtained (HC-J6, Okamoto et al., 1991; and HC-J8, Okamoto et al., 1992a). Comparisons show a similar sequence interrelationship as found in NS-5. HC-J6 differs from HC-J8 by only 23%, while both differ from the other major type by 32-33%. Although different regions of the HCV genome display varying degrees of diversity, the distinction between type and sub-type is consistent. In the core and NS-3 regions differences between types range

from 15-22% and 34-49% respectively, in the NS-4 region variability between HCV types 1 and 2 is 25-27%, with the regions encoding the putative envelope proteins (E1, E2/NS-1) being the most variable with greater than 50% sequence divergence in certain hypervariable regions (Weiner et al., 1991; Hijikata et al., 1991a).

The 3′ untranslated region is highly variable with variation in both sequence and length between different HCV types (Han et al., 1992) and within different clones from the same isolate (Okamoto et al., 1991). However it is possible that the published sequences are not complete and have yet to reach the end of the genome. There is also dispute as to whether a polyadenaline or a polyuridine tract is present (Han et al., 1991; Kato et al., 1990; Chen et al., 1992). By analogy with other positive-sense RNA viruses, it is likely that HCV forms secondary structures at the 3′ end of the genome for initiation of minus-sense RNA synthesis (Han et al., 1992) although the details have yet to be elucidated.

The 5′ non-coding region (5′NCR) or untranslated region (UTR) shows much less variability between HCV types than the coding regions. There are very few nucleotide differences in this region between HCV types, with a maximum sequence divergence of 10%. The sequence conservation of the 5′NCR may reflect functional constraints. *In vitro* studies have implicated the 5′NCR as having a regulatory role in virus replication or initiation of translation (section 3.3.) The HCV 5′NCR has the potential to form stable and complex secondary structures, containing stem loop structures. These structures may be required for virus replication and initiation of

translation and may considerably restrict the degree of sequence variability in the 5'NCR. In a recent study (Simmonds et al.,1993b), comparison of eight HCV sequences revealed five covariant positions within the 17 base paired residues forming one of the stems. This frequency of compensatory substitutions which maintain base pairing would be unlikely to have arisen by chance. In addition, the positions of single or double nucleotide insertions localise to the non-base-paired terminal loop and would therefore have no effect on base pairing within the stem structure. In this way secondary structures for different variants of HCV are highly conserved.

3.6.2. CLASSIFICATION OF HCV VARIANTS

There have been several attempts to classify HCV sequence variants into different types. Phylogenetic analysis of nucleotide sequences amplified in the region of the genome encoding the core protein and parts of the NS3 and NS5 proteins showed that although different degrees of variability were found in various parts of the genome, analysis of each produced phylogenetic trees topologically identical to those obtained upon analysis of complete genome sequences (Chan et al., 1992). Phylogenetic analysis of seventy-six nucleotide sequences, with a world wide distribution, derived from part of the gene encoding the non-structural protein (NS5) indicated that HCV variants could be grouped into six major genotypes. Many of the HCV types comprised a number of closely related subtypes, resulting in a total of eleven genetically distinct viral populations (Simmonds et al., 1993c). A classification system has been proposed that would allow for new types and subtypes to be added

as they are discovered (table 3.2.). In general, the consensus sequence of each type and subtype may be used to identify and classify new HCV variants. The NS5 region is a useful region for virus identification since it is sufficiently variable to allow this distinction to be made. Sequence similarities of less than 72% with any of the consensus nucleotide sequences would indicate the designation of a new type. Those showing sequence similarities of between 75% and 86% with particular variants and less than 72% with others would be assigned as a new subtype.

Enomoto et al., (1990) classified sequences as K1/PT and K2, the first being subdivided along the geographical lines indicated in section 3.6.1., and K2 comprising the K2a and 2b subgroups. Houghton et al., (1991) classified sequences as type I (sequences of USA/European origin), type II (Far East sequences) and type III (sequences of Japanese origin). Any new sequences discovered would then be designated type IV. However, this classification is uneven in that the difference between type I and II sequences in each protein coding region is consistently less than that between I and III. Fewer amino acid substitutions exist between type I and II sequences than between other variants, and type I and II sequences cannot be differentiated in the 5'NCR. Alternative classifications have identified the existence of distinct genotypes but fail to take into account the two-tiered nature of sequence differences (Okamoto et al., 1991; Okamoto et al., 1992b; Mori et al., 1992). The scheme described by Okamoto et al. and Mori et al. describes HCV types I, II, III, IV, V and VI which correspond to types 1a, 1b, 2a, 3a and 3b respectively in the

Table.3.2. COMPARISON OF NOMENCLATURE FOR HCV TYPES

PROPOSED NAME	PUBLISHED EXAMPLE	CHA/ URDEA	CHAN/ SIMMONDS	ENOMOTO	MORI/ OKAMOTO	TSUKIYAMA -KOHARA
1a	HCV-1, -H	I	1a	K-PT	I	nc
1b	HCV-J, -BK	II	1b	K-1	II	I
1c	-	nc†	nc	nc	nc	nc
2a	HC-J6	III	2a	K-2a	III	II
2b	HC-J8	III	2b	K-2b	IV	II
2c	-	III	nc	nc	nc	nc
3a	Ta, E-b1	IV	3	nc	V	nc
3b	Tb	IV	nc	nc	VI	nc
4a	-	nc	4	nc	nc	nc
5a	-	V	nc	nc	nc	nc
6a	-	nc	nc	nc	nc	nc

Proposed nomenclature for published HCV sequences and comparison with existing schemes (CHA/URDEA : Cha et al, 1992; CHAN/SIMMONDS : Chan et al., 1992; Simmonds et al., 1993; ENOMOTO : Enomoto et al., 1990; MORI/OKAMOTO : Okamoto et al., 1992; Mori et al., 1992; TSUKIYAMA-KOHARA : Tsukiyama-Kohara et al., 1991). † nc : sequences not classified by originating authors indicated as "nc".

system proposed by Simmonds et al., 1993c. Cha et al. (1992) described five groups of HCV variants, where I corresponds to 1a, II to 1b, III to 2a, 2b and 2c, IV to 3a and 3b and V to type 5a. These classification systems would be difficult to extend to incorporate types and subtypes as they are discovered (eg: 1c and 2c). Differences between the various schemes have made it difficult to compare results from different laboratories. Table 3.2. shows a comparison of the nomenclature systems for HCV, the system proposed by Simmonds et al. (1993c) is now widely used in the literature and will be the system referred to in subsequent chapters.

3.6.3. PHYLOGENETIC ANALYSIS OF HCV VARIANTS

The discovery of HCV variants (Enomoto et al., 1990; Nakao et al., 1991; Okamoto et al., 1991) that differed markedly in sequence from the original prototype HCV (HCV-1; Choo et al., 1991) and others found in Japanese patients (Kato et al.,1990; Takamizawa et al., 1991) raised the possibility that further HCV variants may exist. A study to examine nucleotide sequence diversity in naturally infected individuals (blood donors) in Scotland (Chan et al., 1992) revealed the existence of a group of 5'NCR sequence variants that differed from all previously published data.

Direct sequence analysis revealed constant as well as variable regions. Six sequences (E-b13 through E-b18) (fig. 3.5) closely resembled HCV-1 (sequence no.1) and the other type 1 sequences listed in table 3.3., while four others (E-b9 to E-b12) were more closely related to "K2" and HC-J6 sequences (nos. 24, 27, 28). However, eight sequences (E-b1 through E-b8) were distinct from the others (fig 3.5.). Sequence

variability within the three groups was considerably less than that which separates them, and no sequence intermediate between the three groups was found. Division of these and previously published sequences into three groups was supported by formal phylogenetic analysis shown in fig 3.6.. This tree shows that the third group is equally distinct from group 1 as is group 2. The group labelled "1" contains sequences of HCV with a worldwide distribution (sequences 1-15; table 3.3), while group "2" contains K2 and J6 sequences first observed in Japan (nos. 24, 27, 28). The corrected distances between sequences within each group were in each case less than 3%. Between groups, they ranged from 9% (between 1 and 3, and between 1 and 2), to 13% between 2 and 3 (Chan et al., 1992). Further analysis in the core, NS3 and NS5 regions confirmed that the new group of sequences were also distant from the other two previously identified groups.

Figure 3.5.

		-255	-235	-215	-195	-175	-156
3	E-b1	GGCGTTAGTA	CGAGTGTCGT	GCAGCCTCCA	GGACTCCCCC	TCCCGGGAGA	GCCATAGTGG	TCTGCGGAAC	CGGTGAGTAC	ACCGGAATCG	CTGGGGTGAC
3	E-b2	...	.....	.....	....C.....	.....	.....	.....	.....	.....	.....
3	E-b3	...	.....	.....	....C.....	.....	.....	.....	.....	.....	.....
3	E-b4	...	.....	.....	....C.....	.....	.....	.....	.....	.....	.....
3	E-b5	...	.....	.....	....C.....	.....	.....	.....	.....	.....	.....
3	E-b6	...	.....	.....	....C.....	.....	.....	.....	.....	.....	.....
3	E-b7		.....	.....	..C.C.....	.....	.....	.....	.....	.....	.....
3	E-b8		.....	.....	....C.....	.....	.....	.....	.....	.....	.....
2	K2a	.....	T.....A.	.....	..C.C.....	.....	.....	.....	.....	.....T.	.C...AA...
2	HC-J6	.....	T.....	A.....	..C.C.....	.....	.....	.....	.....	.....T.	.C...AA...
2	E-b9		T.....	A.....	..C.C.....	.....	.....	.....	.....	.....T.	.C...AA...
2	E-b10		T.....	A.....	..C.C.....	.....	.....	.....	.....	.....T.	.C...AA...
2	E-b11		T.....	A.....	..C.C.....	.....	.....	.....	.....	.....TA	.C..AAA...
2	E-b12	...	T.....	A.....	..C.C.....	.....	.....	.....	.....	.....T.	.C..AAA...
2	K2b-1	.....	T.....A.	.....	..C.C.....	.....	.....	.....	.....	.....TA	.CG..AA...
1	E-b13	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	E-b14	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	E-b15	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	E-b16		T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	E-b17		T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	E-b18		T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	HCV-1	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	Pt-1	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	H77	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	H90	.....	T.....	A.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	GM1	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	GM2	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	J1	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	A1	.....	T.....	.....	....C.....	.....A...	.....	.....	.....	.....T.	.C...AG...
1	S1	.....	T.....	.....	....C.....	.....A...	.....	.....	.....	.....T.	.C...A....
1	T1	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..A....
1	U18/I24	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	HCV-J	.....	T.....T..	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	HCV-BK	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	HC-J1	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...
1	HC-J4	.....	T.....	.....	....C.....	.....	.....	.....	.....	.....T.	.CA..AC...

Figure 3.5. continued

		-155 ▼		-135 ▼		-115 ▼		-95 ▼		-75 ▼	-62 ▼
3	E-b1	CGGGTCCTTT	CTTGGAGCAA	CCCGCTCAAT	ACCCAGAAAT	TTGGGCGTGC	CCCCGCGAGA	TCACTAGCCG	AGTAGTGTTG	GGTCGCGAAA	GGCC
3	E-b2	....	....	....	....	....	....	....	....	....	....
3	E-b3	....	....	....	....	....	....	....	....	....	....
3	E-b4	....	....	....	....	....	....	....	....	....	....
3	E-b5	....	....	....	....	....	....	....	....	....	....
3	E-b6	....	....	....	....	....	....	....	....	....	....
3	E-b7	....	....	....	....	....	......A...	C.........	....	....	....
3	E-b8	....	......A...	....	....	....	....	....	....	....	....
2	K2a	T.........	......TA..	...A...T..	G...G.TC..	....	......A..				
2	HC-J6	T.........	......TA..	...A...T..	G...G.TC..	....	......A...	CTG.......	.....C....	...T......	....
2	E-b9	T.........	......TA..	...A...T..	G...G.CC..	....	......A...	CTG.......	.....C....	...T......	....
2	E-b10	T.........	......TA..	...A...T..	G...G.CC..	....	......A...	CTG.......	.....C....	...T......	....
2	E-b11	T.........	......TA..	...A...T..	GT..G.TC..	....	......A...	CTG......T	.....C....	...T......	....
2	E-b12	T.........	......TA..	...A...T..	GT..G.TC..	....	......A...	CTG......T	.....C....	...T......	....
2	K2b-1	T.........	......TA..	...A...T..	GT..G.TC..	....	......A..				
1	E-b13	....	......T...	....	G..TG..G..	....	......A...	CTG.......	....	....	....
1	E-b14	....	......T...	....	G..TG..G..	....	......A...	CTG.......	....	....	....
1	E-b15	....	......T...	....	G..TG..G..	....	......A...	CTG.......	....	....	....
1	E-b16	....	......TT..	....	G..TG..G..	....	......A...	CTG.......	....	....	....
1	E-b17	....	......TT..	....	G..TG..G..	....	......A...	CTG.......	....	....	....
1	E-b18	....	......T...	....	G..TG..G..	....	....	CTG.......	....	....	....
1	HCV-1	....	......T...	....	G..TG..G..	....	......A...	CTG.......	....	....	....
1	Pt-1	....	......TA..	....	G..TG..G..	....	......A..				
1	H77	....	......TA..	....	G..TG..G..	....	......A...	CTG.......	....	....	....
1	H90	....	......TA..	....	G..TG..G..	....	......A...	CTG.......	....	....	....
1	GM1	....	......TA..	....	G..TG..G..	....	......A...	CTG.......	....	....	...
1	GM2	....	......TA..	....	G..TG..G..	....	......A...	CTG.......	....	....	...
1	J1	....	......TA..	....	G..TG..G..	.......C..	......A..				
1	A1	....	......TA..	....	G..TG..G.C	....	......A...	CTG.......	....	....	....
1	S1	....	......TA..	....	G..TG..G..	....	......A...	CTG.......	....	....	....
1	T1	....	......TA..	....	G..TG..G.C	....	......A...	CTG.......	....	....	....
1	U18/I24	....	......TA..	....	G..TG..G..	....	......A...	CTG.......	....	....	....
1	HCV-J	....	......T...	....	G..TG..G..	....	....	CTG.......	....	....	....
1	HCV-BK	....	......T...	....	G..TG..G..	....	....	CTG.......	....	....	....
1	HC-J1	....	......TA..	....	G..TG..G..	.......C..	......A...	CTG.......	....	....	....
1	HC-J4	....	......T...	....	G..TG..G..	....	....	CTG.......	....	....	....

Figure 3.5. LEGEND.

Comparison of nucleotide sequences in the 5′non coding region from British blood donors (E-b1 through E-b18) with previously published HCV sequences. "." : identity with sequence of E-b1 (top line); nucleotide substitutions indicated. Nucleotide numbering corresponds to that described for the prototype HCV-1 sequence (Choo et al., 1991). Source and citation of published sequences shown in table 3.3.; phylogenetic group indicated in left hand column.

Figure 3.6.

Phylogenetic analysis of the 5'NCR sequences using the maximum likelihood algorithm, shown as an unrooted tree. Symbol ●, nos1 - 18 correspond to blood donor sequences E-b1 through E-b18. Symbol ○, nos 1- 28 correspond to the previously published sequences identified in Table 3.3.

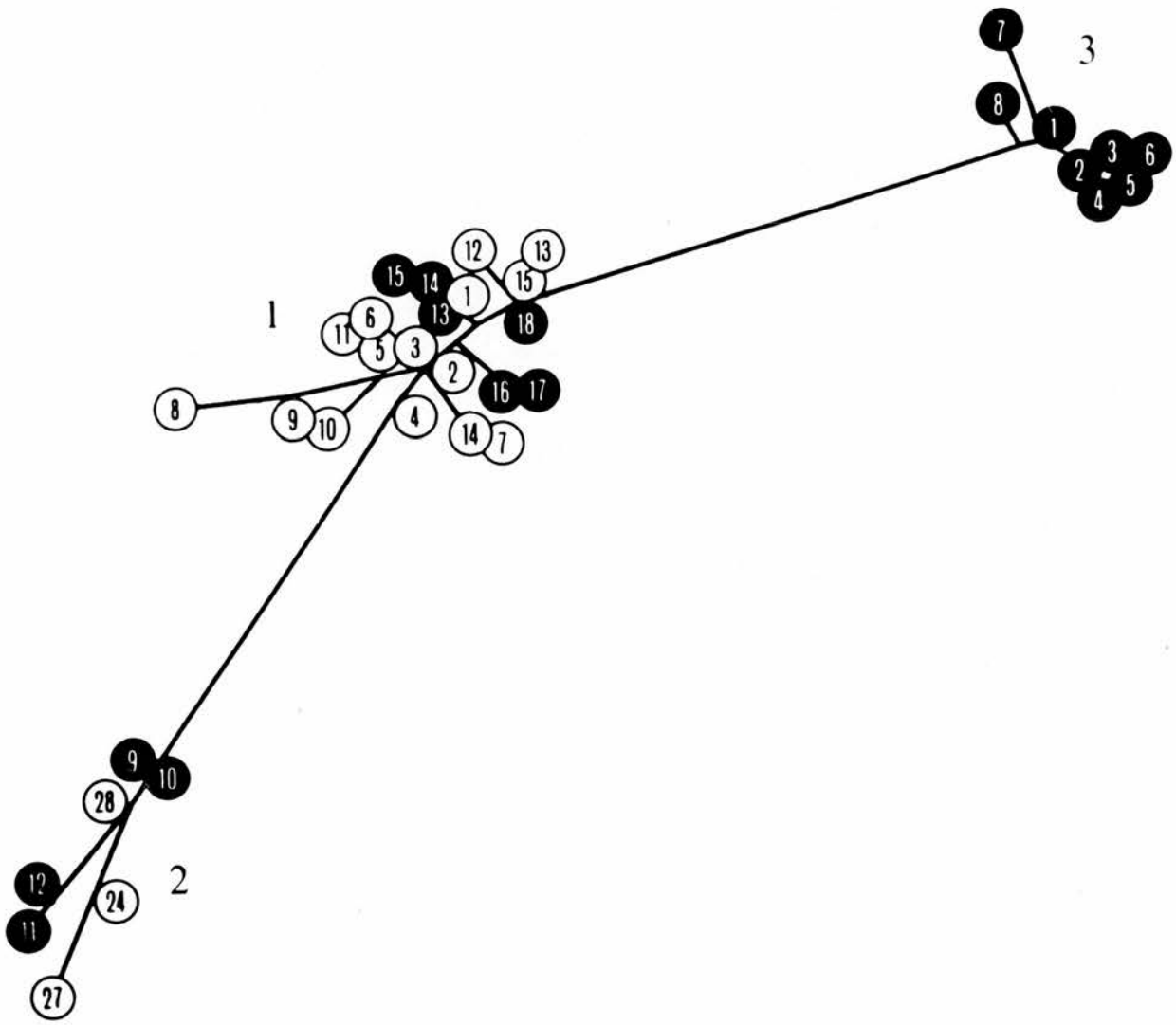


Table 3.3.

SOURCE AND CITATION OF PUBLISHED HCV SEQUENCES

No.	Type	Abbreviation	Geographical Source	Reference
1	1a	HCV-1	U.S.A.	Choo et al. (1991)
2	1a	Pt-1	Japan	Nakao et al. (1991) Enomoto et al. (1990)
3, 4	1a	H77, H90	U.S.A.	Ogata et al. (1991)
5, 6	1a	GM-1,GM-2	Germany	Fuchs et al. (1991)
7	1	J1	Japan	Han et al. (1991)
8	1	A1	Australia	Han et al. (1991)
9	1	S1	S. Africa	Han et al. (1991)
10	1	T1	Taiwan	Han et al. (1991)
11	1	U18/I24	U.S.A/Italy	Han et al. (1991)
12	1b	HCV-J	Japan	Kato et al. (1990)
13	1b	HCV-BK	Japan	Takamizawa et al. (1991)
14,15	1a,b	HC-J1, -J4	Japan	Okamoto et al. (1990b)
16-20	1b	K1, K1- 1-4	Japan	Enomoto et al. (1990)
21	1b	JH	Japan	Kubo et al. (1989)
22	1b	J7	Japan	Takeuchi et al. (1990)
23	1b	T3	Taiwan	Chen et al. (1991)
24-27	2a 2b	K2a, K2a-1, K2b, K2b-1	Japan	Nakao et al. (1991) Enomoto et al. (1990)
28	2a	HC-J6	Japan	Okamoto et al. (1991)
29	2	Clone A	Japan	Tsukiyama-Kohara et al. (1991)

From the published data there is considerable evidence for an overlapping distribution of HCV types in a single geographic area. The existence of different hepatitis C viral types may be related to distinct clinical disease syndromes associated with HCV infection. Differences in the pathogenicity of the different types of the virus may be related to the extent of disease observed and varying response to interferon treatment. The degree of sequence variability found between HCV types would be expected to affect the antigenicity of many of the putative proteins of HCV. In the following chapter (4) the relationship between detection of antibody and HCV viraemia is investigated in the blood donating population. The distribution and the effect of sequence variants in relation to the efficacy of blood donor screening is considered further.

CHAPTER 4

4. BLOOD DONOR SCREENING FOR HEPATITIS C VIRUS

4.1. GENERAL INTRODUCTION

HCV is recognised as the main cause of PT-NANBH worldwide. Relying on the results of antibody screening alone for counselling of donors and elimination of donations from use is problematic since false positive reactions may occur and the relationship between an antibody response and HCV viraemia is not clearly defined. In the following sections the relationship between antibody result and viraemia detected by RT-PCR is examined.

4.1.1. ANTI-HCV ANTIBODY ASSAYS : FIRST GENERATION ASSAYS

The first HCV antigen to become available for antibody screening assays was the 5-1-1 antigen derived from the NS4 gene. Further cloning led to the purification of the c100 antigen from the same region of the genome (location shown in fig 3.1., section 3.3.). This antigen was used to coat microtitre plates with the subsequent development of a radioimmunoassay that specifically detected anti-HCV antibodies in a panel of well characterised serum samples. Using this newly developed assay system, anti-HCV antibodies were detected in patients with chronic PT-NANBH whose blood had proven to be infectious to chimpanzees, and also in the implicated donors to these patients (Kuo et al.,1989). The development of a commercially available enzyme-linked immunoassay (anti-HCV c100 ELISA) enabled the widespread screening of blood donors and anti-HCV testing in certain diagnostic situations.

Several countries in Europe and the United States initiated programs of blood donor screening relying on these first generation assays. However sensitivity and specificity were low, resulting in the reporting of false positive and negative results. Analysis of stored sera from prospective studies showed that the anti-HCV assay had a sensitivity of more than 90% when used to test blood recipients who developed NANBH or in their implicated donors (van der Poel et al., 1990a). However, when used to test random, non-implicated donors, only 30% to 50% of the donors with repeatedly reactive serum could be detected by a secondary test such as the recombinant immunoblot assay (RIBA). One reason for false negative results may be that a response to the c100 antigen is sometimes not detected for up to three months following infection (Alter et al., 1989). In addition some polymerase chain reaction (PCR) proven infections have been shown not to produce an antibody response to c100 (Weiner et al., 1990). It has also been demonstrated that the c100 RIBA/c100 EIA combination is less sensitive than the c100 EIA alone (van der Poel et al.,1990a,b). Another factor contributing towards false negative results may be a difference in antigenicity due to sequence variablity between different types of hepatitis C virus.

At this time the only alternative test available was the RIBA with the 5-1-1 and c100 antigens immobilised on nitrocellulose strips. This assay therefore does not provide independent confirmation of a positive result as both antigens are derived from the NS4 region of the genome and is considered to be a supplemental test. To provide confirmation of a positive result, antigens derived from a different region of the

genome should be used in the assay system. Alternatively, direct detection using hybridization or reverse transcription followed by PCR (RT-PCR) should be carried out in a highly conserved region of the genome such as the 5′ non-coding region (NCR).

4.1.2. ANTI-HCV ANTIBODY ASSAYS : SECOND GENERATION ASSAYS

In response to these problems a second generation anti-HCV EIA was subsequently developed which detects antibodies against part of the core polypeptide (c22-3) and part of the NS3 region (c33c), in addition to anti-c100. A second generation RIBA (RIBA-2) was developed containing five recombinant antigens, 5-1-1 and c33c produced in *Escherichia coli*, and c100-3, c22-3 and SOD (superoxide dismutase, a control) produced in yeast. Donations are classified as positive which show significant reactivity with two or more HCV antigens (band strength of 1+ to 4+), or indeterminate when reactivity is with one antigen only. Comparison of the sensitivity of the second generation EIA with the c100 EIA and RIBA-2 confirmed the increased sensitivity of the second generation assays. A retrospective study on PT-NANBH patients showed that 26 weeks post transfusion, seroconversion was detected in 100% of patients using the 2nd generation EIA while only 67% were detected with the c100 EIA (Bresters et al. 1992). In addition, early detection of antibody was observed with the second generation assay (van der Poel et al., 1992; Wang et al. 1992). The RIBA-2 gave an increased sensitivity of HCV antibody detection in blood donors, and transfusion recipients with PT-NANBH and patients with chronic NANBH (Ebling et al., 1990; Marcellin et al., 1991). The use of second generation tests has

significantly lowered the risk of transmission of HCV infection via blood transfusion (Donahue et al., 1992).

4.1.3. ANTI-HCV ANTIBODY ASSAYS : THIRD GENERATION ASSAYS

In an attempt to further increase the sensitivity and specificity of tests, an EIA containing antigen derived from the NS5 region of the HCV genome in addition to c100-3, c33c and c22-3, found in the second generation tests was developed. The RIBA-3 utilizes three recombinant antigens and two synthetic peptide bands. The recombinant antigens are c33c produced in *Escherichia coli*, in addition to NS-5 and SOD produced in yeast. The synthetic peptide bands consist of peptides derived from the core and NS4 region of the HCV genome, c22-3 and c100-3 respectively. Third generation assays have only recently become available. It is anticipated that the RIBA-3 will provide additional information on donations determined as indeterminate by RIBA-2.

4.1.4. DIFFICULTIES ASSOCIATED WITH SCREENING FOR HCV INFECTION

It has been difficult to predict the prevalence of HCV in blood donors in the UK since there is a shortage of data on the incidence of post-transfusion hepatitis. A study carried out in North London suggested a rate of post-transfusion hepatitis which was much lower that that reported in other parts of the world (Contreras et al., 1991). It was estimated that transmission occured at a rate of 1 in 1,300 donors in the UK. In Scotland testing of plasma pools suggested a frequency of < 1 PCR-positive

donation/1,000 in the local donor population (Simmonds et al., 1990c). Concerns about the effectiveness of the second generation tests for anti-HCV screening followed the reporting of data showing continued transmission of HCV by screened donations (Esteban et al., 1990; Japanese Red Cross Non-A Non-B Hepatitis Research Group, 1991). Although screening of blood donors in an attempt to prevent PT-NANBH has greatly reduced the incidence of transmission cases there remain donations that transmit HCV but which are seronegative or indeterminate in commercial serological tests (Esteban et al., 1990; van der Poel et al., 1991b). Some of these false negative serological results may be the result of infection by extreme sequence variants of HCV that elicit an antibody response that has limited or no cross reactivity with the peptide antigens used in the serological assays. Such failures may sometimes be due to infectious donations that are collected in the period between infection and seroconversion in the donor. In addition, the humoral response to HCV antigens may be abnormally restricted or reduced in immunocompromised individuals. In the following sections 4.2.3.- 4.2.4 the relationship between detection of antibody and HCV viraemia in the blood donating population is considered. An HCV typing assay is described in section 4.3. which enables the HCV genotype to be determined without the need for direct sequence analysis. Using such an assay the HCV genotype may be determined on a large number of samples in a short period of time. The newly developed HCV typing assay was used to investigate whether sequence heterogeneity might influence the effectiveness of serological screening for HCV in blood donors and the extent of nucleotide sequence diversity and distribution of HCV types worldwide in naturally infected individuals (described in section 4.4.).

4.2. CORRELATION OF RIBA REACTIVITY AND THE PRESENCE OF HCV RNA

4.2.1. INTRODUCTION

In September 1991 routine screening of all blood donations in the UK for HCV antibody was implemented. Screening of low risk populations such as blood donors produces a significant number of indeterminate results with the RIBA-2 assay, which gives rise to the problem of how to interpret this result in relation to HCV viraemia and counselling of the donor. Like the first generation HCV tests, the second generation tests may produce false positive reactions (Kolho et al., 1992; Bresters et al., 1992; Garson et al., 1992). A similar problem of obtaining confirmation of the initial screening results (EIA) exists as the supplemental tests (RIBA) rely on antigens derived from the same regions of the genome. Information on the relationship between RIBA-2 and RIBA-3 positive and indeterminate results and HCV viraemia was obtained by reverse transcription followed by the polymerase chain reaction (RT-PCR) to detect HCV RNA in the donating population in Scotland. An accurate value for the prevalence of HCV infection in Scottish blood donors could then be obtained and evaluation of the role of PCR as a confirmatory test in HCV screening be assessed.

4.2.2. SAMPLES

(1) RIBA-2 TESTING

A total of 103,203 volunteer blood donations collected in Scotland and Northern Ireland in the first three months after the introduction of HCV screening (between September and November 1991) were used in the RIBA-2 study. No repeat donations should be present as three months is the minimum period between which whole blood may be donated. Blood donor samples were screened at the Regional Transfusion Centres (RTCs) for antibody to HCV using second-generation HCV ELISA, (Abbott GmBH, Wiesbaden-Delkenhein, Germany; Ortho Diagnostic Systems, Raritan, NJ.). Glasgow, Edinburgh, Aberdeen and Belfast RTCs used the Abbott anti-HCV kit and referred 275 repeatedly reactive donations from screening 89,708 donations (0.31%), while the Inverness and Dundee Centres used Ortho kits and referred 65 donations from screening 13,495 donations (0.48%).

(2) RIBA-3 TESTING

All RIBA-2 indeterminate samples from the first 3 months of screening were tested by RIBA-3. In addition, between October 1992 and April 1994 a total of 2787 repeatedly reactive donations from screening for anti-HCV by ELISA were referred to Ruchill Hospital by RTCs. Only new donors and those previously screened giving a RIBA-2 indeterminate and PCR negative result were included in this number since all RIBA-2 positive and PCR positive donors would be excluded from further donation.

Referred donations were tested by RIBA-2 or RIBA-3 (Chiron Corporation, Emeryville, California) by Dr. Brian Dow and staff at The SNBTS Microbiology Reference Unit, Regional Virus Laboratory, Ruchill Hospital, Glasgow. Donations that were RIBA positive (reactivity with two or more HCV antigens) or indeterminate (reactivity with only one antigen), were tested for virus RNA by RT-PCR (chapter 2, sections 2.5. and 2.6.)

4.2.3. RESULTS

(1) SUPPLEMENTARY TESTING : RIBA-2

Of the 340 referred donations, 77 (22.6%) were positive by RIBA-2, 130 (38.2%) were indeterminate, and the remaining 133 (39.1%) were negative. All RIBA-2 positive samples exhibited reactivity to c33c and c22-3 bands, except for one sample which only reacted to the 5-1-1 and c100-3 bands (table 4.1.). Of the 130 samples indeterminate by RIBA-2, most showed only weak reactivity (scored 1+ or 2+) to one of the four antigens, 5-1-1, c100-3, c33c and c22-3 with few strong bands (of 4+) seen except to the c22-3 antigen (table 4.2.).

(2) CONFIRMATORY TESTING : PCR

During the study period, 65/77 (84.4%) of RIBA-2 positive samples were PCR positive (table 4.1.), compared to only 7 (5.5%) of the 130 RIBA-2 indeterminate samples (table 4.2.). None of the indeterminate samples with reactivity to either the c100-3 or 5-1-1 antigens were PCR positive, but virus RNA was detected in one

Table 4.1. RIBA-2 CONFIRMED PATTERNS AND PCR RESULTS OF REFERRED SAMPLES

RIBA-2 Pattern				Total	
5-1-1	**c100-3**	**c33c**	**c22-3**	**Number**	**PCR+**
+	+	+	+	35	30(86%)
+	-	+	+	2	1(50%)
-	+	+	+	13	10(77%)
-	-	+	+	26	24(92%)
+	+	-	-	1	0
			Total no.	77	65(84%)

Table 4.2. PCR RESULTS OF RIBA-2 INDETERMINATES ACCORDING TO BAND INTENSITY

RIBA-2 band	**No.**	**Intensity**	**PCR+**
5-1-1	1	1+	0
c100-3	24	1+	0
	14	2+	0
	2	3+	0
	1	4+	0
c33c	6	1+	0
	2	2+	1
	1	3+	0
c22-3	30	1+	0
	17	2+	0
	16	3+	0
	16	4+	6
Total no.	130		7

sample with reactivity to c33c (scored as 2+) and in 6 samples that were strongly reactive (4+) with the c22-3 antigen. The 133 RIBA-2 negative samples were tested by PCR in pools of 10, but all were PCR negative. The average virus titre in a unit of plasma has been reported to be between 5 X 10^5 and 10^8 HCV copies/ml (Garson et al., 1992;). This range was confirmed for Scottish blood donors by titration of ten samples (section 2.9.). The limit of sensitivity of this RT-PCR assay was 200 copies/ml (Simmonds et al., 1990c). Therefore, introducing a dilution factor of 1:10 by pooling samples would still enable the detection of all but a few infected donations with extremely low titres of virus.

(3) SUPPLEMENTARY TESTING : RIBA-3

Testing all 130 RIBA-2 indeterminate samples by RIBA-3 produced 14 positive, 28 indeterminate and 88 negative results. Two samples showed reactivity with NS-5 only, and 3 were classified as positive due to additional NS-5 reactivity.

Of the 2787 referred donations, 159 (5.7%) were positive by RIBA-3, 643 (23%) were indeterminate, and the remaining 1985 (71.3%) were negative. Sixty-seven and 51 RIBA-3 positive samples showed reactivity to 4 and 3 bands respectively, all but 7 had reactivity to the c33c and c22-3 antigens. Forty-one samples (26%) showed reactiviy to 2 bands only, 17/41 (41%) were reactive to both c33c and c22-3 with 7/41 (17%) and 14/41 (34%) showing reactivity with c33c and c22-3 respectively (plus one other band) (table 4.3.). Of the 643 indeterminate samples 23%, 23%, 18%

and 36% showed reactivity to c100-3, c33c, c22-3 and NS-5 antigens alone respectively.

(4) CONFIRMATORY TESTING : PCR

Testing all new donors and those with previously RIBA-2 indeterminate RT-PCR negative results by RIBA-3 identified 159 RIBA-3 positive donors, 114 (71.7%) of which were also RT-PCR positive (table 4.3.). Five out of 643 (0.78%) RIBA-3 indeterminate samples were RT-PCR positive. Of the RIBA-3 indeterminate samples, 1 sample that showed reactivity to the c100-3 antigen was RT-PCR positive which is unusual. One other sample which showed reactivity to the c33c antigen (with a score of 2+), and RT-PCR positive was identified. This sample was from the same individual that had shown similar reactivity in the RIBA-2 in the first 3 months of screening. Of the remaining indeterminate samples 3/16 were RT-PCR positive and showed reactivity to the c22-3 antigen (table 4.4.).

Table 4.3. RIBA-3 CONFIRMED PATTERNS AND PCR RESULTS OF REFERRED SAMPLES

RIBA-3 Pattern				Total	
c100-3	**c33c**	**c22-3**	**NS5**	**Number**	**PCR+**
+	+	+	+	67	57(85%)
+	+	+	-	36	26(72%)
+	+	-	+	3	3(100%)
+	-	+	+	4	4(100%)
-	+	+	+	8	8(100%)
			Total no.	51	41(80%)
+	+	-	-	6	2(33%)
+	-	+	-	11	3(27%)
+	-	-	+	3	0
-	+	+	-	17	11(65%)
-	+	-	+	1	0
-	-	+	+	3	0
			Total no.	41	16(39%)
			Total no.	**159**	**114(72%)**

Table 4.4. PCR RESULTS OF RIBA-3 INDETERMINATES ACCORDING TO BAND INTENSITY

RIBA-3 band	No.	Intensity	PCR+
c100-3	49	1+	0
	44	2+	0
	43	3+	0
	15	4+	1
c33c	44	1+	0
	32	2+	1
	41	3+	0
	28	4+	0
c22-3	41	1+	0
	33	2+	0
	27	3+	0
	16	4+	3
NS5	102	1+	0
	74	2+	0
	48	3+	0
	6	4+	0
Total no.	**643**		**5(0.78%)**

4.2.4. DISCUSSION

Of the 340 referred samples from Scottish and Northern Ireland Blood Transfusion Centres 72 (21.2%) samples (ie: ELISA repeat reactive samples) were shown to have HCV RNA in the plasma. The majority of RIBA-2 positive samples were PCR positive (84%). This finding is consistent with other studies that have demonstrated the RIBA-2 assay is efficient in detecting donations reactive in first generation EIAs that were viraemic (Garson et al., 1992; van der Poel et al., 1991c;). In contrast, the majority of RIBA indeterminate donations failed to react by PCR and only 7 out of 130 (5.4%) were found to contain HCV RNA. All of these samples exhibited a single band in the RIBA-2 indicating reactivity to the core antigen (c22-3), except for one sample with reactivity only to the c33c antigen. Twelve of the 77 RIBA-2 positive samples (15.6%) were PCR negative. These samples and an unknown number of indeterminate samples may contain HCV antibody and may represent donors that have recovered from HCV infection. Alternatively, PCR negative samples may be a result of the PCR assay not being sufficiently sensitive. Fluctuations in the levels of viraemia may be another possible explanation for these antibody positive and seemingly PCR negative donations.

The confirmatory strategy used by the SNBTS involves the use of RIBA-2, a supplementary test incorporating the same antigens as used in the screening tests, followed by PCR. The use of RIBA-2 as a confirmatory test is therefore less than ideal. A confirmatory test should be at least as sensitive as the screening test and use antigens from a different source. For example, confirmation of reactivity in HIV-1

recombinant antigen ELISAs is made by using Western blots of whole virus lysate and containing additional antigens. Information from PT-NANBH patient samples have confirmed that the RIBA-2 system is less sensitive than the screening EIAs in the early stages of seroconversion (pers. comm. Dr. B. Dow; van der Poel et al., 1992). However, no PCR positives were found in the ELISA reactive, RIBA-2 negative samples in this study. Although the pooling strategy and the sensitivity of the RT-PCR would not permit detection of donations with virus titres of less than 200 copies per ml, it is unlikely that significant numbers of specific HCV antibody containing donations failed to be detected because of any comparative insensitivity of RIBA-2.

Testing 130 RIBA-2 indeterminate samples by RIBA-3 reduced the numbers reacting as positive and/or indeterminate to 42. RIBA-3 testing of samples over the initial 3 month period would have reduced the number sent for RT-PCR testing from 207 to 119. It is not possible to compare the RIBA-2 results from the first 3 months of testing with RIBA-3 results obtained after this date as a different donor population is being tested. However, since the RIBA-3 incorporates improved recombinant antigens and synthetic peptides from 4 different regions of the HCV genome it is anticipated that an increase in sensitivity will be observed. The importance of the NS-5 component is uncertain, none of the RIBA-3 and RT-PCR positive samples that showed reactivity to 2 antigens were reactive with the NS-5 antigen and none of the indeterminate samples that were reactive to this antigen alone were RT-PCR positive. While reactivity to the c33c and c22-3 antigens appears to have a predictive value

for RT-PCR positivity the greater sensitivity provided by the RIBA-3 should reduce further the number of samples that need to be tested by RT-PCR.

The prevalence of HCV infection in the blood donor population in Scotland and Northern Ireland is 0.07% (no. of PCR positive, RIBA-2 positive or indeterminate samples/no. of donations screened; 72/103,203) which is relatively low in comparison with other countries (Stevens et al.,1990; Richards et al., 1991; Alberti et al., 1991). The prevalence of HCV infected donors is expected to fall in the future as HCV infected individuals are excluded from further donation. Extrapolation of these figures to the whole of the UK would suggest that aproximately 3,000 HCV infected donors would have been identified in the first year of testing.

This study indicates that the only method of determining whether an HCV antibody positive sample is likely to transmit HCV is to carry out a direct test for HCV viraemia using the PCR. However, the use of the RT-PCR as a routine screen for HCV adds significantly to the cost, time and is difficult to standardise. Exclusion of all RIBA-2 positive donations from further use would eliminate 77 out of 65 PCR positive donations and thus provide an improvement in the safety of blood and blood products. However, the RIBA-2 indeterminate donation represents a significant problem. Most are likely to be nonspecific reactions since they are PCR negative and may have non-specific antibody reactivity with the antigens used in the assays. A small number probably represent specific antibody following HCV infection and some (7 out of 130) may be viraemic. Where funding or access to PCR analysis is

limited, routine testing by RT-PCR of RIBA-2 or RIBA-3 indeterminate samples would be the most efficient approach. Alternatively, a combination of alternative antigen EIAs and/or RIBA-3/line immunoassays could reduce the number of samples that would require PCR testing (Dow et al., 1993). However, irrespective of the combination of tests used, PCR remains the only method of identifying a viraemic donor and the only independent confirmation of viraemic HCV infection.

4.3. DEVELOPMENT OF AN HCV TYPING ASSAY

4.3.1. INTRODUCTION

There is little information on the effect of virus sequence variation on the effectiveness of screening for antibody to HCV. The original anti-HCV enzyme immunoassay (EIA) used a recombinant protein, c100-3, derived from the NS-4 region of the viral genome as the solid phase antigen in an indirect ELISA, and proved capable of detecting most, but not all, donations implicated in the transmission of HCV to recipients (Houghton et al., 1991; Mori et al., 1992). Second generation EIAs include recombinant antigens from the core and NS-3 regions of HCV and are capable of much higher detection rates of HCV antibody in blood donors and haemophiliacs (Bresters et al., 1992; Craxi et al., 1991). However, some HCV infected blood donors may not be detected because of the delay between primary infection and seroconversion for antibody to HCV. However, infection with HCV genotypes divergent in sequence from HCV type 1 might elicit antibodies that do not cross-react with the antigens used in current screening assays. In order to investigate this possibility, an RFLP typing system was developed and used to screen HCV infected blood donors. This information was then used to correlate serological response with HCV genotype. This assay also led to the identification of novel HCV genotypes (section 4.3.3.(2)) and allowed their geographical distribution to be identified (section 4.4.). Sequence information on all HCV variants would assist in the development of serological tests that are equally sensitive in detecting all HCV types, not only type 1.

4.3.2. DEVELOPMENT OF A RFLP ASSAY TO DETECT HCV TYPES 1 TO 3.

Several different methods have been developed for the typing of HCV variants: direct sequence analysis (Cha et al., 1992; Chan et al.,1992; Kato et al.,1990), slot-blot hybridization analysis of RT-PCR products using cDNA probes specific to each HCV genotype (Takada et al., 1993) and PCR amplification using type specific primers that are designed to match only viral sequences of a defined HCV type and will fail to amplify sequences of other types (Okamoto et al., 1992). An alternative method is the non-selective amplification of virus cDNA using conserved primers followed by restriction endonuclease cleavage and electrophoresis of the DNA fragments to detect restriction fragment length polymorphisms (RFLP) (Nakao et al., 1991). The coding regions of the HCV genome are so variable that reliable amplification of all HCV types with the same set of PCR primers is difficult. For this reason, typing methods based on the analysis of amplified DNA are only reliable if carried out in the highly conserved 5'NCR. It is possible that the RFLP system could be extended to identify subtypes of the major genotypes since there are certain consistent differences in the 5' NCR of subtypes within a single genotype. The first combination of enzymes described were concerned with the identification of HCV type 1 and type 2 (Nakao et al., 1991) specifically. A recently discovered third HCV type (Chan et al., 1992) would remain undetected by this system.

Predicted cleavage patterns produced by all common restriction enzymes of blood donor and haemophiliac sequences obtained in a study by Chan et al.(1992) and

sequences reported elsewhere (Choo et al.,1991; Okamoto et al.,1991; Nakao et al.,1991; Kato et al.,1990; Takamizawa et al.,1991; Ogata et al.,1991; Fuch et al.,1991; Han et al.,1991) were computed with standard sequence-analysis software (Devereux et al.,1984). Restriction enzymes were identified that produced patterns that were clearly resolved by polyacrylamide gel electrophoresis. Using the combination of enzymes HaeIII and RsaI in one reaction along with ScrFI in a separate reaction it was possible to differentiate HCV types 1, 2 and 3 from each other by the unique cleavage patterns obtained (see chapter 2, section 2.10. for experimental details).

A total of 78 HCV sequences in the 5'NCR were compared using phylogenetic methods (see chapter 2, section 2.8.2.), and assigned into HCV types 1, 2 and 3. A total of 7 distinct cleavage patterns were predicted for the restriction endonucleases HaeIII and RsaI in combination (fig.4.1a.). All of the type 1 sequences obtained in this and the study described in chapter 3, section 3.6.3., as well as all published type 1 sequences produced restriction patterns a and b, type 2 sequences patterns c, d and e, and all type 3 sequences produced patterns f and g (fig.4.1a. and fig.4.2a.).

ScrFI independently differentiates all known type 1 sequences (pattern A, B) from type 2 (C, D, E and F) and from type 3 (G; fig. 4.1b. and fig. 4.2a). The two adjacent ScrFI sites present in all but two of the sequence variants (pattern B) could not be simultaneously cleaved, so patterns A and B are for practical purposes equivalent, and are referred to as A/B.

Figure 4.1a.

PREDICTED CLEAVAGE PATTERNS FOR HaeIII/RsaI DIGEST

a	44		58		114/5				9	26
b	102				114/5				9	26
c	44	12	46		58	56			9	26
d	44	12	46		114				9	26
e	56		46		114				9	26
f	33	69			114				9	26
g	33	23	46		114				9	26

Figure 4.1b.

PREDICTED CLEAVAGE PATTERNS FOR ScrFI DIGEST

A	53	15	48	9	32	94				
C	53	15	57		32	94				
D	53	15	48	41	94					
E	53	15	183							
F	53	15	48	135						
G	53	15	41	16	126					

Figure 4.2a.

Predicted combinations of RFLP patterns obtained from cleavage with HaeIII/RsaI and ScrFI associated with sequences of HCV types 1 to 3.

		ScrFI						
		A/B	C	D	E	F	G	Type
HaeIII /RsaI	a	**9**	-	-	-	-	-	1
	b	**35**	**1**	-	-	-	-	
	c	-	-	**5**	-	-	-	
	d	-	-	**1**	**2**	-	-	2
	e	-	-	**1**	-	**1**	-	
	f	-	-	-	-	-	**15**	3
	g	-	-	-	-	-	**8**	
Type		1		2			3	

Figure 4.2b.

Observed RFLP patterns of HCV variants amplified from 100 blood donor samples, showing inferred HCV type.

		ScrFI						
		A/B	C	D	E	F	G	Type
HaeIII /RsaI	a	**3**	-	-	-	-	-	1
	b	**47**	-	-	-	-	-	
	c	-	-	**3**	-	-	-	
	d	-	-	**1**	**3**	-	-	2
	e	-	-	**1**	**2**	-	-	
	f	-	-	-	-	-	**38**	3
	g	-	-	-	-	-	**2**	
Type		1		2			3	

4.3.3. RESULTS

(1) SCOTTISH BLOOD DONORS

Amplified and radiolabelled 5'NCR sequences from a total of 100 PCR-positive, RIBA confirmed or indeterminate subjects were typed by RFLP with both ScrFI and HaeIII/RsaI. Observed combinations of restriction patterns and therefore the inferred HCV type for each sample are shown in fig 4.2b. No sample produced restriction patterns incompatible with the combinations of published or sequences derived as part of this study (fig. 4.2a). Types 1 and 3 are the predominant variants in Scotland (table 4.5.); 50% of donors are infected with type 1, 40% with type 3, while type 2 accounts for the remaining 10%.

(2) IDENTIFICATION OF HCV VARIANTS

Using the RFLP system described the distribution of HCV types worldwide was explored in order to carry out a search for new variants outside the existing classification. Blood donor samples from nine different countries were available (described in section 4.4.2.) and previously published sequences from another worldwide survey (Bukh et al., 1992).

RFLP analysis of plasma samples of Egyptian blood donors indicated that all infections were of the same type, HCV type 1. In a previous study on Scottish blood donors (McOmish et al., 1993a), those infected with type 1 showed broad reactivity with all four antigens in the RIBA-2, while those infected with HCV types 2 and 3

Table 4.5.

FREQUENCY OF INFECTION WITH HCV TYPES 1 TO 3 IN SCOTTISH BLOOD DONORS

HCV TYPE	FREQUENCY (%)
1	50/100 (50%)
2	10/100 (10%)
3	40/100 (40%)
Total PCR pos	100/113 (88%)
Total PCR neg	13/133 (12%)

showed reactivity restricted to the NS4 derived antigens (c100-3 and 5-1-1) only (see also section 4.4.3(2). and 4.4.4.). RIBA-2 results for the Egyptian blood donor samples varied, broad and restricted serological reactivity with the antigens in the RIBA-2 was observed, which would be expected with donors infected with the three different HCV genotypes, not with type 1 alone, the genotype determined by RFLP. To further investigate this apparently unusual serological profile, direct sequence analysis was carried out on all PCR positive donations from Egyptian donors. One sample from a blood donor in Holland (IN-26; sequence no.7) produced a 1a electropherotype with the HaeIII/RsaI digest which is not common. This sample was investigated further also by direct sequence analysis.

Sequences of the 5'NCR (fig.4.3.) are presented as a phylogenetic tree (fig.4.4.). This analysis reveals sequences 1-10 form a separate cluster from variants previously typed as 1, 2, and 3. This new group of sequences was assigned as HCV type 4 (Simmonds et al., 1993b). Mean nucleotide distances within type 4, and between type 4 and the other HCV types in the 5'NCR are comparable to those previously described for intra and inter-type distances for other types (table 4.6a.). The majority of sequences within type 4 form a close group, but sequences 11, 12, and 13 differ considerably from any of the known HCV types. Comparison with published sequences from Zaire (shown as hollow squares) (Bukh et al., 1992) reveals that they group closely with type 4 sequences described here. However, the relatively small numbers of substitutions that differentiated these sequences from those of type 1 and 3 meant that an analysis of more variable regions of the genome was neccessary.

RNA from three representative type 4 variants (EG 29, 33, 21; corresponding to 5'NCR sequences nos. 1-3) was amplified using primers in the core region of HCV polyprotein (chapter 2, section 2.6., table 2.1.). All three sequences differed considerably at both the nucleotide and amino acid level from HCV types 1 to 3 (fig.4.5. and 4.7.). Phylogenetic analysis of HCV sequences in the core region gave further support to the proposal that the sequences designated type 4 did in fact constitute a new type of HCV (fig.4.6). The unrooted maximum likelihood tree for core sequences suggests that the three Egyptian-derived sequences form an independent phylogenetic group since they are separated by long branch-lengths from the other sequences. Nucleotide distances between type 4 and types 1 to 3 were comparable to those that exist between the three known HCV types (table 4.6b.). Although most of the nucleotide sequence changes were silent, there were between 4 and 9 amino acid differences between the type 4 variants and other types (fig 4.7.).

Figure 4.3. 5′NON-CODING REGION SEQUENCES OF NOVEL GENOTYPES

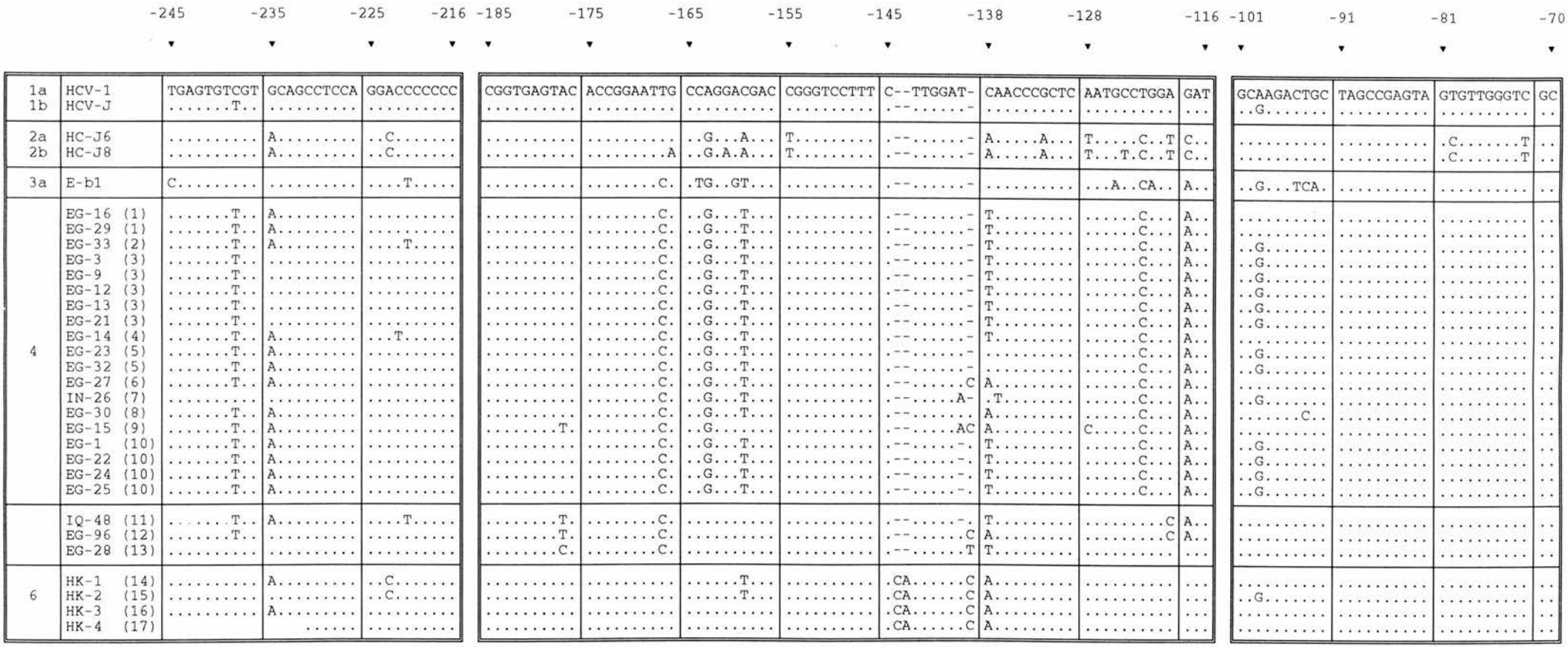


Figure 4.3. LEGEND

Comparison of divergent HCV sequences with representative type 1, 2 and 3 sequences in variable regions of the 5′ NCR. Sequences from -254 to -245, -214 to -185, -114 to -101 and -68 to -61 identical to prototype sequence, and not shown to save space. (.) : sequence identity with HCV-1; (-) : gap introduced in sequences to preserve alignment; ( ) : sequence not determined. Figures in parentheses number each non-identical sequence used for phylogenetic analysis.

Figure 4.4. LEGEND

Phylogenetic analysis of the 5'NCR region using the maximum likelihood method (DNAML), shown as an unrooted tree. Symbol ● nos. 1 - 17 sequences numbered as in figure 4.3; previously published sequences numbered as in table 1 of Chan et al., (1992). Scottish blood donor sequences: Symbol O Eb-1 - Eb-12 numbered 51 - 62. Only non-identical sequences are shown in tree; eg. Sequence 1 corresponds to those found in samples EG-16 and EG-29 etc (Figure 4.3.). Symbol □ : Published sequences (Bukh et al., 1992) from Zaire; Symbol o : sequences from South Africa; Symbol • : sequences obtained elsewhere in the world. All branch lengths are shown to scale.

Figure 4.4. PHYLOGENETIC TREE OF 5′ NON-CODING REGION SEQUENCES SHOWN IN Fig.4.3.

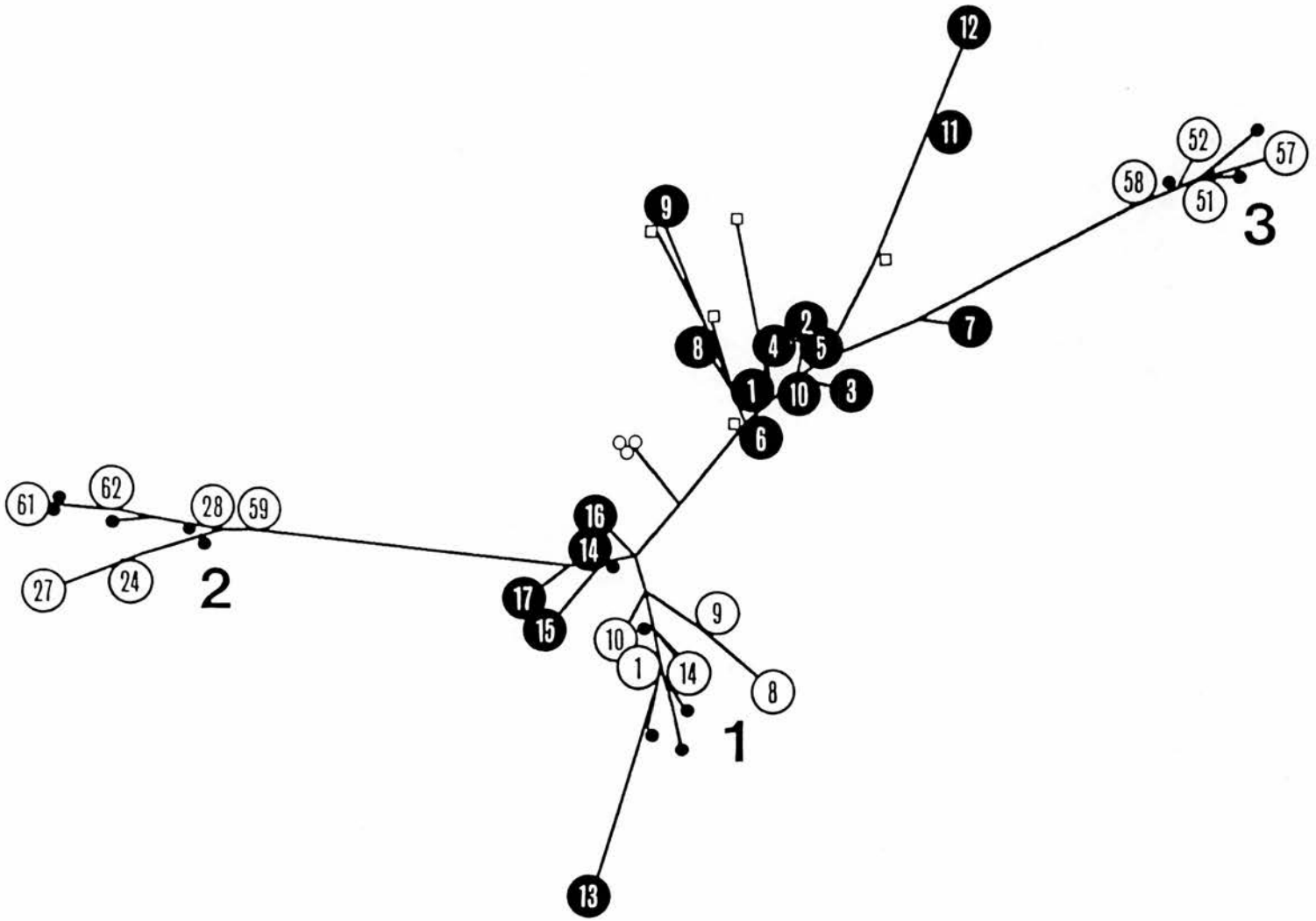


Table 4.6a.

MEAN NUCLEOTIDE DISTANCES BETWEEN THE FOUR MAJOR GROUPS OF SEQUENCES VARIANTS IN THE 5' NC REGION

	n*	1†	2	3	4
1	14	0.018			
2	13	0.091	0.020		
3	9	0.095	0.145	0.012	
4	13	0.054	0.089	0.080	0.022

* Numbers of non-identical sequences in each group.
† Sequences 14-17 (HK- 1-4) that group with type 1 sequences excluded from comparison (see section 4.3.3(2).).

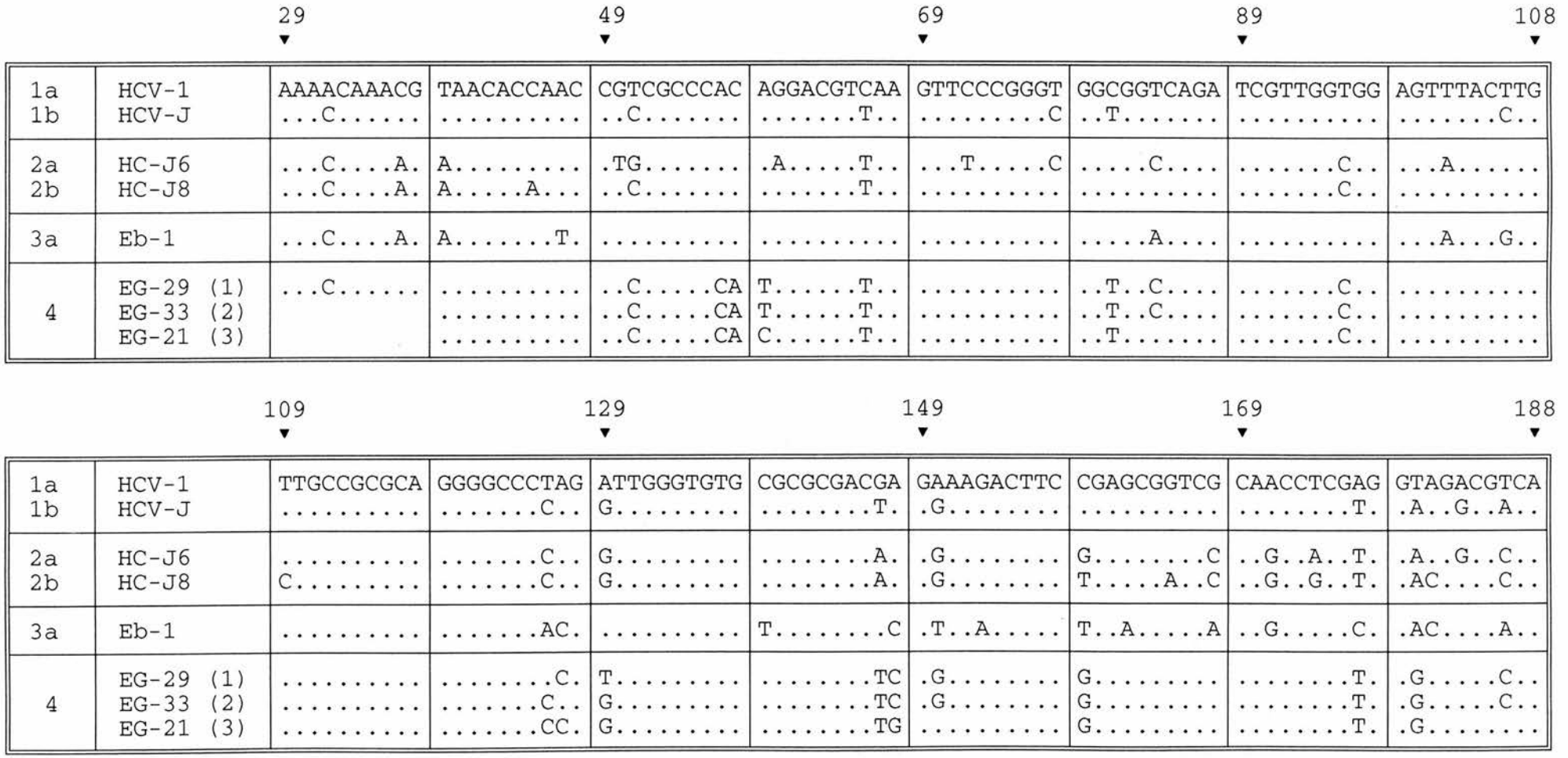
Table 4.6b.

NUCLEOTIDE DISTANCES BETWEEN HCV SEQUENCE VARIANTS IN THE CORE REGION

	n*	1a	1b	2a	2b	3a	4†
1a	6	0.023					
1b	5	0.086	0.036				
2a	1	0.223	0.205	0			
2b	1	0.193	0.206	0.130	0		
3a	1	0.151	0.180	0.219	0.220	0	
4	3	0.161	0.157	0.222	0.196	0.223	0.042

* Numbers of non-identical sequences in each group.
† Sequences from EG-28, -33 and -21 that form new group of sequences in the 5'NCR.

Figure 4.5.



		29		49		69		89	108
1a	HCV-1	AAAACAAACG	TAACACCAAC	CGTCGCCCAC	AGGACGTCAA	GTTCCCGGGT	GGCGGTCAGA	TCGTTGGTGG	AGTTTACTTG
1b	HCV-J	...C.....	.....	..C.....	.....T..	C	..T.....	.....	.....C..
2a	HC-J6	...C....A.	A.....	.TG.....	.A....T..	...T....C	.....C....	.....C..	...A.....
2b	HC-J8	...C....A.	A.....A...	..C.....	.....T..	.....	.....	.....C..	.....
3a	Eb-1	...C....A.	A.....T.	.....	.....	.....	.....A....	.....	...A...G..
4	EG-29 (1)	...C.....	.....	..C.....CA	T.....T..	.....	..T..C....	.....C..	.....
	EG-33 (2)		.....	..C.....CA	T.....T..	.....	..T..C....	.....C..	.....
	EG-21 (3)		.....	..C.....CA	C.....T..	.....	..T.....	.....C..	.....

		109		129		149		169	188
1a	HCV-1	TTGCCGCGCA	GGGGCCCTAG	ATTGGGTGTG	CGCGCGACGA	GAAAGACTTC	CGAGCGGTCG	CAACCTCGAG	GTAGACGTCA
1b	HCV-J	.....	.....C..	G.....	.....T.	.G.....	.....	.....T.	.A..G..A..
2a	HC-J6	.....	.....C..	G.....	.....A.	.G.....	G.....C	..G..A..T.	.A..G..C..
2b	HC-J8	C.....	.....C..	G.....	.....A.	.G.....	T.....A..C	..G..G..T.	.AC....C..
3a	Eb-1	.....	.....AC.	.....	T.....C	.T..A.....	T..A.....A	..G.....C.	.AC....A..
4	EG-29 (1)	.....	.....C.	T.....	.....TC	.G.....	G.....	.....T.	.G.....C..
	EG-33 (2)	.....	.....C..	G.....	.....TC	.G.....	G.....	.....T.	.G.....C..
	EG-21 (3)	.....	.....CC.	G.....	.....TG	.....	G.....	.....T.	.G.....

Figure 4.5. continued

		189 ▼		209 ▼		229 ▼		249 ▼	269 ▼
1a 1b	HCV-1 HCV-J	GCCTATCCCC A.....	AAAGCTCGTC .....C.	GGCCCGAGGG	CAGGACCTGG T.....	GCTCAGCCCG	GGTACCCTTG	GCCCCTCTAT	GGCAATGAGG .....C....
2a 2b	HC-J6 HC-J8	...C.....T ...C.....G	....A...G. ....A...G.	.CT..ACT.. .CT..ACC..	..AAT..... ..A.T.....	.GAA.A..A. .GAA....A.	.A.....C.. .A..T.....	......A..C ......G..C	..G..C.... ..A..C....
3a	Eb-29	.....	.....G....	..AG...A..	....T.....	.....	.....G..	.....	..T..C....
4	EG-29 (1) EG-33 (2) EG-21 (3)	A.....A A.....A	..G..G.... ..G..G.... ..G..G....	.AT..... .AT..... .AT.....	A...T..... A...T..... A...T.....	..A..A..A. ..A.....A. ..A.....A.	.A..T..A.. .A..T..A.. .A.TT..A..	...T..T..C ...T..T..C	..T..... ..A..A

Figure 4.5. LEGEND : Comparison of nucleotide sequences in the core region of three type 4 variants with published sequences of HCV types 1, 2, and 3.

Figure 4.6.

PHYLOGENETIC TREE OF NUCLEOTIDE SEQUENCES

OF PART OF THE CORE REGION

Phylogenetic analysis of nucleotide sequences of part of the core region (positions 13 to 380) using DNAML, shown as an unrooted tree. Sequences numbered as in Fig 4.4.. Sequence 30 corresponds to isolate HC-J8 (Okamoto et al., 1992). All branch lengths are shown to scale.

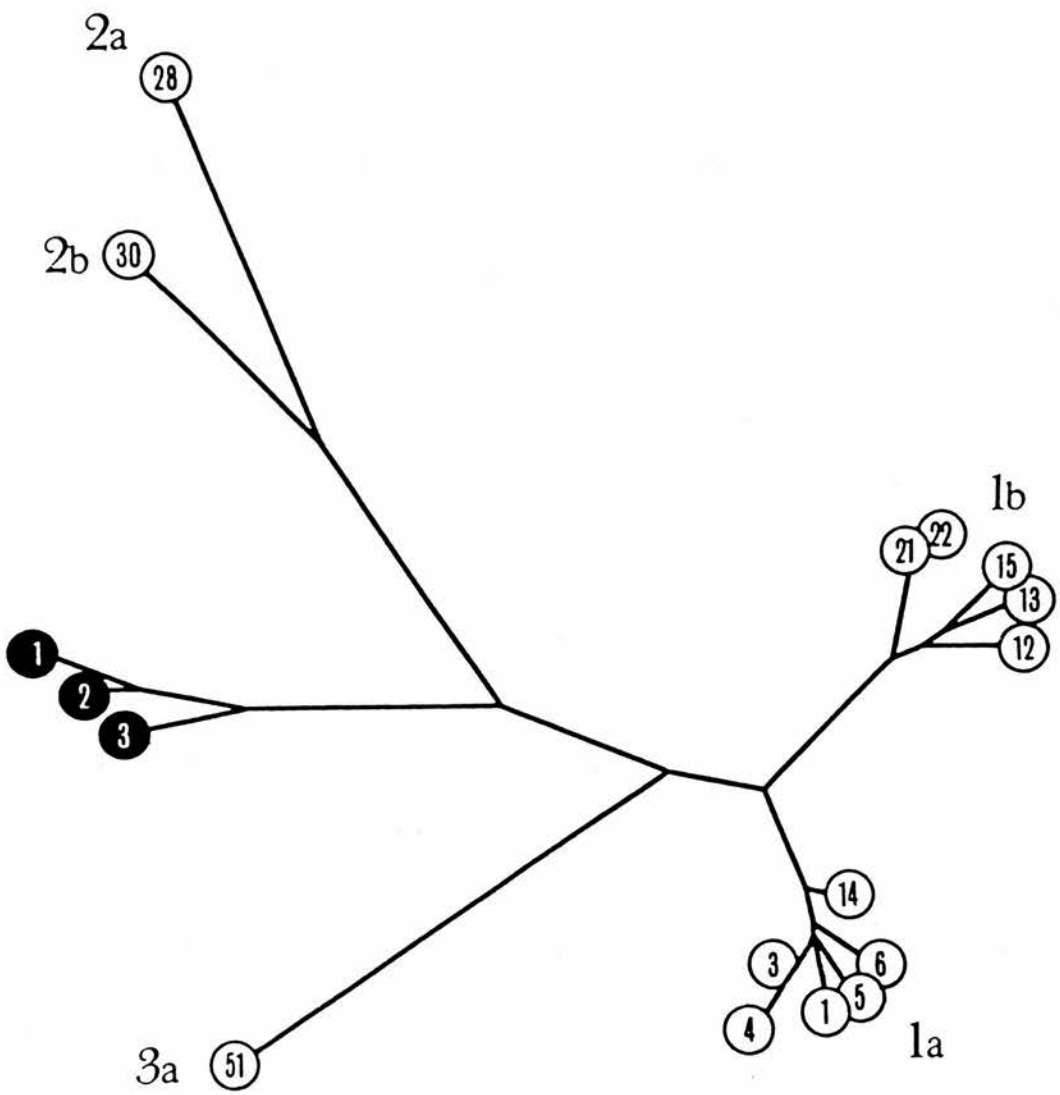


Figure 4.7.

1a	HCV-1	PKPQKKNKRN	TNRRPQDVKF	PGGGQIVGGV	YLLPRRGPRL	GVRATRKTSE	RSQPRGRRQP	IPKARRPEGR	TWAQPGYPWP	LYGNE
1b	HCV-J	....R.T...	....	....	....	....	....	....	....	.....
2a	HC-J6	....R.T...	....	....	....	....	....	...D..ST.K	S.GK......	.....
2b	HC-J8	....R.T...	....	....	....	....	....	...D..ST.K	S.GK......	.....
3a	Eb-1	A...R.T...	.I........	....	.V........	..C.......	....	......S...	S.........	.....
4	EG-29,33	.R.....	.....M....	....	....	....	....	......S...	S.........	.....
	EG-21	...	.....T....	....	....	.....G....	....	......S...	S.....F...	.....

Figure 4.7. LEGEND : Comparison of amino acid sequences in the core region of three type 4 variants with published sequences of HCV types 1, 2, and 3.
Single letter amino acid codes used.

RFLP analysis carried out on the blood donor samples from Hong Kong showed that the majority of HCV infections were of HCV type 1. However the remaining samples displayed an RFLP pattern that was similar to that expected for a 2d electropherotype with the HaeIII/RsaI digest but showed a slightly larger band in the 114bp fragment. Direct sequence analysis was carried out on 4 representative samples (sequences 14-17 in fig.4.3. and fig.4.4.). All four 5'NCR sequences from Hong Kong blood donors and EG-28 and EG-96, showed a single base insertion between position -138 and -137. In addition the Hong Kong sequences had a two base insertion 5 bp upstream between position -144 and -143 (numbering as in the HCV-1 sequence; see fig 4.3.). Apart from these insertions, the Hong Kong sequences differed little from type 1 5'NCR sequences, and cluster relatively closely to the main group of type 1 sequences in the phylogenetic tree (nos. 14-17; fig.4.4.). Sequence data obtained by Dr. S-W. Chan in the NS-5 region confirmed that these samples from Hong-Kong also represent a new HCV type, designated type 6 (Simmonds et al., 1993b).

Comparison of these newly identified HCV types with other published sequences (Buhk et al., 1992; Simmonds et al.,1993b,c) revealed a number of other sequences that appeared to be of ambiguous status. Two sequences, EG-96 (12) and EG-28 (13) are linked to the other sequences by long branch lengths (fig 4.4), in addition to sequence IQ-48 (Simmonds et al., 1993b). Three sequences from South African patients (hollow circles fig 4.4.; Buhk et al., 1992) appear to be distinct from those of any of the other HCV types and have been classified as HCV type 5 (Simmonds et al., 1993c).

4.3.4. MODIFICATION OF THE RFLP ASSAY TO DETECT HCV TYPES 1 TO 6

Sequence analysis in the NS-5 region of HCV amplified from plasma of Egyptian and Hong Kong blood donors revealed a relatively homogeneous group of novel sequence variants which were as distinct from HCV types 1, 2, and 3 as these types were from each other. These new sequences were designated HCV type 4 and 6 respectively. Another group of variants, found in plasma samples from South Africa (Buhk et al., 1992), were designated HCV type 5. By using the combination of restriction enzymes HaeIII/RsaI and ScrFI it is possible to identify HCV types 1, 2, and 3. However, HCV types 4 and 5 would be indistinguishable from HCV type 1 using this combination of enzymes. Type 1 and type 4 (and type 3) sequences differ in the number of HinfI sites present. Type 4 sequences showed a U->C change at position -166 that creates a novel HinfI site that is absent in all type 1 (and type 2) sequences. Using the combination of HaeIII/RsaI followed by ScrFI/HinfI the bands produced and the nomenclature system used broadly follows that described in section 4.3.2., but with additional patterns for the two novel genotypes 4 and 6. For simplicity, the two patterns with ScrFI/HinfI which are not distinguishable (A and B) by electrophoresis are referred to as A. Similarly, a rare C->T change found in a type 3 and a type 4 sequence at position -220 (eg. E-b1, Chan et al., 1992; EG-33, Simmonds et al., 1993b) creates a new HinfI site, but the position of cleavage is 3 base pairs (bps) downstream from a conserved ScrFI (or MvaI site). As these two sites could not be simultaneously cleaved, the polymorphism would not affect the overall pattern of

bands. Since there are no other variable HinfI or ScrFI sites in type 3 and 4 sequences, a single letter (G, H) has been assigned to each respectively.

Using the combination HaeIII/RsaI, patterns designated a and b are predicted for published type 1, type 4 and type 5 sequences, while patterns designated c, d and e were uniquely associated with type 2 (fig.4.8a.). Patterns f and g should be produced by all known type 3 sequences, and pattern h by type 6. The latter pattern differs from pattern d (type 2) by the two insertions (total 3 bp) in the 114 bp fragment. This size difference can be readily resolved by polyacrylamide gel electrophoresis of the cleaved DNA.

The second set of restriction enzymes (ScrFI/ HinfI) produces different bands for five of the six HCV genotypes (fig.4.8b.). For type 6 sequences, a 3 bp insertion in the 35 bp fragment (pattern I) distinguishes them from type 1 sequences (pattern A; 32 bps). With neither combination of enzymes is it possible to distinguish type 5 from type 1 sequences. The type 5 sequences can be distinguished from type 1 by replacing ScrFI (GGnCC) with MvaI (GG[A/T]CC) to identify an A/G polymorphism at position -162, and a U/C polymorphism at position -121. Further studies using MvaI/HinfI indicate that this combination can be used to entirely replace the ScrFI/HinfI combination for identification of all six genotypes (section 4.4.6.), as suggested by analysis of predicted fragment patterns of the published sequences (fig.4.8a.and 4.8c.). Out of a total of four possible electropherotypes, all type 1

sequences produce pattern A, and type 5 produces pattern D. RFLP gels are shown in chapter 2, sections 2.4. and 2.5..

Figure 4.8a.

a	44		58		114/5		9	26
b	102				114/5		9	26
c	44	12	46	58		56	9	26
d	44	12	46	114			9	26
e	56		46	114			9	26
f	33	69		114			9	26
g	33	23	46	114			9	26
h	44	12	46	117			9	26

	HCV GENOTYPE					
	1	2	3	4	5	6
a	9	-	-	14	-	-
b	60	-	-	4	4	-
c	-	9	-	-	-	-
d	-	10	-	-	-	-
e	-	2	-	-	-	-
f	-	-	21	-	-	-
g	-	-	9	-	-	-
h	-	-	-	-	-	5

Figure 4.8b.

A	53	15	48	9	32	94
C	53	15	57		32	94
D	53	15	48	41		94
E	53	15	183			
F	53	15	48	135		
G	53	15	41	16	126	
H	53	15	41	7	9	32/3	94
I	53	15	48	9	35	94

	HCV GENOTYPE					
	1	2	3	4	5	6
A	68	-	-	-	4	-
C	1	-	-	-	-	-
D	-	11	-	-	-	-
E	-	9	-	-	-	-
F	-	1	-	-	-	-
G	-	-	30	-	-	-
H	-	-	-	18	-	-
I	-	-	-	-	-	5

Figure 4.8c.

A	53	63	41	94
B	53	63	44	94
C	53	56	142/3	
D	53	198		

HCV GENOTYPE					
1	2	3	4	5	6
69	-	-	-	-	-
-	-	-	-	-	5
-	-	30	18	-	-
-	21	-	-	3	-

Figure 4.8. LEGEND

PREDICTED ASSOCIATION OF DIFFERENT CLEAVAGE PATTERNS OF

THE 5′ NCR WITH SEQUENCES OF HCV TYPES 1-6.

Figure 4.8a. : Cleavage with HaeIII/RsaI

Figure 4.8b. : Cleavage with ScrFI/HinfI

Figure 4.8c. : Cleavage with MvaI/HinfI

Location of cleavage sites indicated by vertical lines in the left hand box.

The position of 1 or 2 base pair insertions found in some 5′ NCR sequences represented by cross-hatching.

Expected size of fragments shown in base pairs.

The numbers of published sequences of each genotype associated with each pattern is shown in the right hand box.

4.4. GEOGRAPHICAL DISTRIBUTION OF HEPATITIS C VIRUS GENOTYPES IN BLOOD DONORS

4.4.1. INTRODUCTION

The six major genotypes of HCV have distinct nucleotide sequences in the 5'NCR (Buhk et al.,1992; Simmonds et al.,1993), and so viruses can be typed by RFLP analysis of DNA amplified from this region of the viral genome by reverse transcription of RNA followed by PCR (McOmish et al., 1993a; Nakao et al., 1991). Developing a typing assay based on RFLP requires considerable amounts of comparative sequence data, as there is also some intratypic sequence variability that may lead each genotype to show more than one electropherotype. Furthermore, assays may need to be modified as more HCV genotypes are discovered.

The distribution of the six recognised genotypes in 447 anti-HCV positive volunteer blood donors from nine different countries; Scotland, Finland, the Netherlands, Australia, Egypt, Hungary, Japan, Hong Kong and Taiwan was examined. Serological reactivities to the structural and non-structural HCV type 1 antigens in the recombinant immunoblot assay (RIBA-2) assay of samples from donors infected with different genotypes were assessed in parallel.

4.4.2. SAMPLES

Samples from blood donors from Scotland, Egypt (expatriates working in Riyadh, Saudi Arabia), Finland, Netherlands, Hungary, Australia, and Hong Kong were available from routine 2nd Generation anti-HCV ELISA screening (Ortho or Abbott). Samples from Japan were screened by anti-HCV passive haemagglutination assay (HCV-PHA) using red blood cells coated with PHCV-34 and PHCV-31 antigens (Dainabot Co. Ltd., Abbott, Japan). Samples from Taiwan were screened using a second generation ELISA with HCV antigens equivalent to c22-3, c33c and c100-3. All screening assays were carried out in the country of origin.

Donations from Finland, Netherlands, Hungary, Australia, Egypt that were repeatedly reactive on screening by Abbott (Abbott GmBH, Wiesbaden-Delkenheim, Germany) or Ortho (Ortho Diagnostics Systems, Raritan, NJ), 2nd generation assays were tested further by the RIBA-2 (Chiron Corporation, Emeryville, CA.) (table 4.7.). Samples that were confirmed positive or indeterminate were shipped frozen to Edinburgh for further analysis by polymerase chain reaction (PCR). In Hong Kong, the Abbott Matrix assay was used for supplementary testing. Samples reactive on screening from Japanese donors that showed titres of $> 2^{12}$ in the HCV-PHA assay were referred to Scotland, while those from Taiwanese donors were referred without prior confirmatory testing. In interpreting the RIBA-2 results, reactivity with the 5-1-1 and c100-3 antigens, and no other bands, were considered as an indeterminate result. Both antigens are derived from the same region of NS-4 and could be reacting with the same epitope in both proteins.

4.4.3. RESULTS

(1) HCV TYPING

Amplified sequences from each of the 447 PCR-positive donations were cleaved with two sets of restriction endonucleases (HaeIII/RsaI and ScrFI/HinfI). A wide range of electrophoretic patterns were found in the study samples, but almost all could be assigned to those predicted for published sequences (n=443; table 4.8.). In no cases were there incompatible combinations of RFLP pattern between the two sets of enzymes (fig. 9a.), and all 443 sequences could be assigned as types 1 or type 5, 2, 3, 4, and 6. As sequences of genotype 5 produce the same RFLP patterns with both sets of restriction endonucleases as type 1 (fig. 4.8a. and 4.8.b.), a third cleavage reaction with MvaI/HinfI was carried out on the 252 samples showing the aA or bA electropherotypes (fig. 4.9b.). All but one sample showed the A electropherotype indicating type 1, while the exception showed the D pattern consistent with a type 5 sequence.

Types 1, 2 and 3 were found in the three western European countries surveyed and in Australia, with types 1 and 3 being similar in prevalence and type 2 forming approximately 20% of the total (table 4.9.). One Dutch donor was infected with a variant that showed an RFLP pattern with ScrFI/HinfI normally associated with type 4; this sample was sequenced and found to be similar to those obtained from the Middle East and Egypt (section 4.3.4.) (Simmonds et al., 1993b), Zaire (Bukh et

Table 4.7.
SUPPLEMENTARY TESTING OF DONATIONS REPEATEDLY REACTIVE UPON SCREENING BY SECOND GENERATION EIAs

Country	Donors Screened[a]	Supplementary test[b]	n[c]	RIBA-2 (PCR pos) Confirmed[d]	RIBA-2 (PCR pos) Indet.[e]	No. PCR positive (%)[f]	% infected donors[g]
Scotland	302,231	RIBA-2	510	203 (129)	307 (15)	144 (28%)	0.07%
Australia	20,000	Abbott (2 bead)	48	27 (22)	21 (2)	24 (50%)	0.12%
Finland	137,000	RIBA-2	75	15 (10)	60 (2)	12 (17%)	0.01%
Netherlands	321,400	RIBA-2	34	30 (27)	4 (4)	31 (91%)	0.01%
Hungary	9,707	RIBA-2	73	48 (44)	25 (3)	47 (64%)	0.53%
Egypt	264	RIBA-2	33	23 (19)	10 (0)	19 (58%)	8.7%
Taiwan	5,914	n.d.[h]	100	99 (93)	1 (0)	93 (93%)	1.7%
Japan	4,400	n.d.	44	44 (40)	0 (0)	40 (91%)	1.1%
Hong Kong	29,000	Abbott (Matrix)	39	37 (37)	2 (0)	37 (95%)	0.13%
Total no.			**956**	**526 (421)**	**430 (26)**	**447 (47%)**	

[a] Donor samples from Scotland and Finland were part of a consecutive series, for the Netherlands, Hong Kong and Australia, an estimate was made of the number of donations from which the samples were derived.
[b] Abbott (2 bead): Two bead supplementary assay (Abbott)
[c] n : number of EIA-positive samples reactive in RIBA-2.
[d] RIBA-2 confirmed (significant reactivity to two or more HCV recombinant antigens.
[e] RIBA-2 indeterminate (reactivity to single HCV antigen).
[f] Samples either positive or indeterminate by RIBA-2 were further tested by PCR: HCV type was determined on all PCR positive samples.
[g] Approximate incidence of infection obtained by dividing the number of confirmed infected donors (RIBA-2 positive or RIBA-2 indeterminate/PCR positive) by number of donations screened.
[h] Not done

Table 4.8.

DISTRIBUTION OF ELECTROPHEROTYPES AMONGST DONORS FROM DIFFERENT COUNTRIES

	ELECTROPHEROTYPE[a]											
COUNTRY	**aA**	**bA**	**cD**	**dD**	**eD**	**dE**	**eE**	**fG**	**gG**	**aH**	**bH**	**hJ**
Scotland	4	63	10	2	3	4	2	48	8	-	-	-
Australia	1	12	1	-	1	1	-	8	-	-	-	-
Finland	-	3	-	-	-	5	-	4	-	-	-	-
Netherlands	-	18	2	3	-	2	-	5	-	-	1	-
Hungary	-	45	-	-	-	-	-	-	-	1	-	-
Egypt	-	-	-	-	-	-	-	-	-	12	5	-
Japan	-	31	6	2	-	1	-	-	-	-	-	-
Taiwan	1	52	16	5	7	6	6	-	-	-	-	-
Hong Kong	1	21	-	-	-	1	-	-	-	-	-	12
Total	**7**	**245**	**35**	**12**	**11**	**20**	**8**	**65**	**8**	**13**	**6**	**12**

[a] First letter (lower case; a-h) indicates cleavage pattern observed upon digestion with HaeIII/RsaI (Fig.4.8a.). The second letter (upper case; A-J) refers to the cleavage pattern with ScrFI/HinfI (Fig.4.81b.).

Figure 4.9a.

ScrFI/HinfI

HaeIII /RsaI	A	C	D	E	F	G	H	J	
a	8	-	-	-	-	-	13	-	1,
b	244	-	-	-	-	-	6	-	4,5
c	-	-	35	-	-	-	-	-	
d	-	-	12	20	-	-	-	-	2
e	-	-	11	8	-	-	-	-	
f	-	-	-	-	-	65	-	-	3
g	-	-	-	-	-	8	-	-	
h	-	-	-	-	-	-	-	12	6
	1,	5		2		3	4	6	

Figure 4.9b.

MvaI/HinfI

HaeIII /RsaI	A	B	C	D	
a	8	-	-	-	1,
b	244	-	-	1	4,5
c	-	-	-	-	
d	-	-	-	-	2
e	-	-	-	-	
f	-	-	-	-	3
g	-	-	-	-	
h	-	-	-	-	6
	1	6	3,4	2,5	

Fig. 4.9a. and 4.9b. LEGEND

Observed combinations of cleavage patterns with HaeIII/RsaI (a-h; left margin) with ScrFI/HinfI (A-J; top margin) and MvaI/HinfI (A-D; top margin) for identification of genotypes 1 - 6 in the 443 blood donors in the study. Specific genotypes associated with cleavage patterns shown in right and lower margins (see Fig.4.8.). For MvaI/HinfI, only those samples showing RFLP pattern a or b with HaeIII/RsaI were tested further with MvaI/HinfI.

al.,1992), and Burundi (Stuyver et al.,1993) and which had previously been assigned to the type 4 genotype (Simmonds et al., 1993b,c; Stuyver et al.,1993). Upon checking of donor records, this individual was found to have originated from Indonesia and may have acquired infection there. The one sample identified by RFLP as type 5 (bA, D with MvaI/HinfI) originated from Australia; direct nucleotide sequence analysis confirmed its identity as type 5, as it was identical to the 5'NCR sequences of the previously identified type 5 variants SA1, SA3, SA7 and SA11 (Buhk et al., 1992; Simmonds et al., 1993c). This donor was of French/Polish ancestry. Her only admitted risk factor for HCV infection was blood transfusion in France. No history of travel to South Africa was reported. The distribution of HCV genotypes was markedly different in Hungary where all but one were infected with type 1. The one exception produced an RFLP pattern consistent with type 4 (aH), an identification also confirmed by direct sequence analysis.

Type 1 was also the most frequent found genotype in countries in the Far East, although these countries differed from many of those in Europe by the complete absence of HCV type 3. Approximately a quarter of donors from Hong Kong were infected with a novel HCV type, designated type 6 (Buhk et al., 1992; Simmonds et al., 1993b,c). A completely different distribution of HCV infection was found in Egypt, where all donors who could be typed (17/19) were found to be infected with HCV type 4 (table 4.9.).

Table 4.9. PREVALENCE OF HCV TYPES IN DIFFERENT DONOR POPULATIONS

COUNTRY	n[1]	HCV GENOTYPE (%) 1	2	3	4	5	6
Scotland	144	67 (47%)	21 (14%)	56 (39%)	0	0	0
Australia	24	12 (50%)	3 (13%)	8 (33%)	0	1 (4%)	0
Finland	12	3 (25%)	5 (42%)	4 (33%)	0	0	0
Netherlands	31	18 (58%)	7 (23%)	5 (16%)	1 (3%)	0	0
Hungary	47	46 (98%)	0	0	1 (2%)	0	0
Egypt	19[2]	0	0	0	17 (90%)	0	0
Taiwan	93	53 (57%)	40 (43%)	0	0	0	0
Japan	40	31 (77%)	9 (23%)	0	0	0	0
Hong Kong	37[3]	22 (59%)	1 (3%)	0	0	0	12 (32%)
Totals	**447**	**252**	**86**	**73**	**19**	**1**	**12**

[1] Number of samples PCR-positive and typed by RFLP.
[2] Two donors were infected with variants that could not be classified as type 1 - 6.
[3] Two samples showed evidence of mixed infection with types 1 and 6.

Only two of the donors showed evidence of infection with more than one genotype. Both of these were from Hong Kong and showed a mixed pattern of type 1 and type 6 electropherotypes. Co-infection with two genotypes was confirmed by limiting dilution of cDNA amplification of single molecules by nested PCR (Simmonds et al., 1990a). RFLP analysis of DNA amplified from five single molecules yielded 3 type 1 and 2 type 6 sequences from one of the two donors who showed this type of mixed pattern.

Two of the nineteen samples from Egypt produced anomalous RFLP patterns producing bands with HaeIII/RsaI larger than any of those predicted in fig.4.8a.. Sequence analysis confirmed that a A->U or an A->C change at position -177 eliminated the normally well conserved RsaI site to produce bands of 44, 173 (ie. 58 + 115; see pattern a), 9 and 26 bps, or 217 (ie. 102 + 115; see pattern b), 9 and 26 bps. Although the first sequence showed substantial similarity to type 4 variants, they have both been left unassigned, sequence data in the NS-5 region is currently being obtained to allow formal classification.

(2) SEROLOGY

Irrespective of the geographic origin of the donor, sera from those infected with HCV type 1 showed broad reactivity with all four antigens in the 2nd generation RIBA-2 with a high mean score in all cases (fig.4.10; table 4.10.). The antibody response elicited by infection with HCV types 2, 3, 4 and 6 showed reactivity that was largely restricted to c33c and c22-3 antigens (fig. 4.10.) (HCV type 5 is represented by 1

sample only in this study, serological reactivity of type 5 infected donors is considered separately in section 4.4.4.). Reactivity to 5-1-1 was found in 79%, 17%, 7%, 42% and 33% in donors infected with types 1, 2, 3, 4 and 6, respectively, and to c100-3 in 78%, 34%, 33%, 58% and 58% respectively (table 4.10). There was a significant difference between the proportion of type 1 samples reactive with 5-1-1 compared with the other four genotypes either individually (type 2, $p<0.0001$; type 3, $p<0.0001$; type 4, $p<0.001$ and type 6, $p<0.001$) or collectively ($\chi^2=176$, 4 degrees of freedom on a 2 x 2 contingency table; $p<0.0001$). There were also significant differences between type 1 and type 2, 3, 4 and 6 in the frequency of reactivity to c100-3 ($p<0.0001$, $p<0.0001$, $p<0.05$, $p\approx0.1$). Between type 1 and the four genotypes together there was a highly significant difference ($\chi^2=82$, 4 degrees of freedom, $p<0.0001$).

Only 26 out of 430 RIBA-indeterminate samples were PCR positive (6%) compared with 421 PCR-positive out of 526 RIBA-2 confirmed samples (80%) (table 4.10.). All indeterminate donations reactive with the NS-4 derived proteins c100-3 or 5-1-1 were PCR negative, while a significant proportion of those showing an indeterminate reaction with c33c or c22-3 were PCR positive (2/24, 23/339 respectively). Approximately 20% of donors infected with type 3 were reactive with c22-3 only, compared with 3% of those infected with type 1 ($\chi^2=23$; $p<0.0001$). However, sequence variability was not the only cause of restricted serological reactivity, as many of the samples that failed to react with c100-3 and 5-1-1 were obtained from donors infected with type 1 (10 of 430 RIBA-indeterminate samples and 33 of the

166 samples reactive with c22-3 and c33c only). Whether this restricted pattern of reactivity is associated with proximity to seroconversion is currently being assesed by testing previous donations and follow-up samples from the donors. Retrospective testing of stored samples has shown that the type 1-infected donor with reactivity to c33c only (table 4.10.) showed a similar serological profile for at least two years (data not shown).

Figure 4.10.

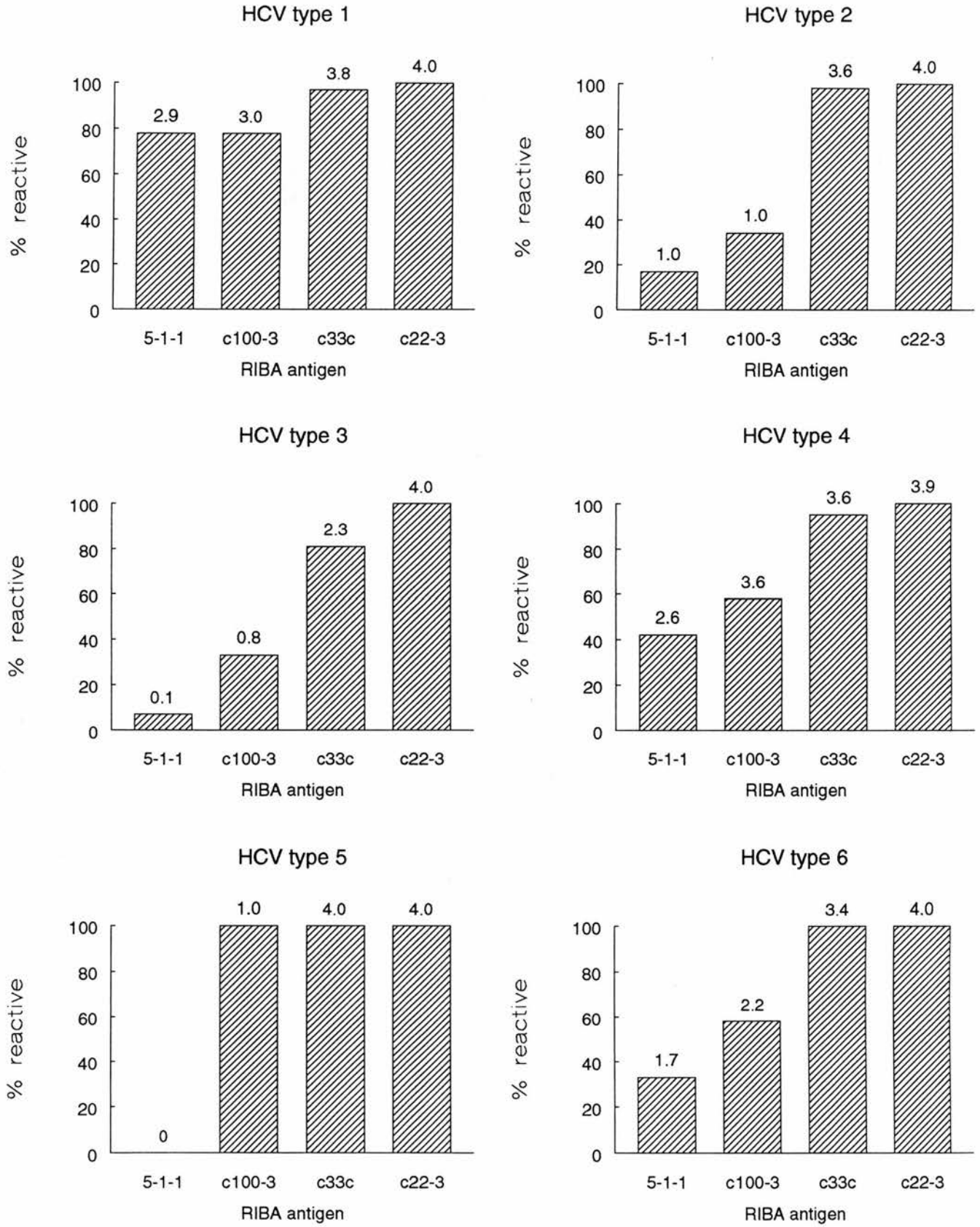

HCV type 1
% reactive
100
80
60
40
20
0
2.9
3.0
3.8
4.0
5-1-1
c100-3
c33c
c22-3
RIBA antigen
HCV type 2
% reactive
100
80
60
40
20
0
1.0
1.0
3.6
4.0
5-1-1
c100-3
c33c
c22-3
RIBA antigen
HCV type 3
% reactive
100
80
60
40
20
0
0.1
0.8
2.3
4.0
5-1-1
c100-3
c33c
c22-3
RIBA antigen
HCV type 4
% reactive
100
80
60
40
20
0
2.6
3.6
3.6
3.9
5-1-1
c100-3
c33c
c22-3
RIBA antigen
HCV type 5
% reactive
100
80
60
40
20
0
0
1.0
4.0
4.0
5-1-1
c100-3
c33c
c22-3
RIBA antigen
HCV type 6
% reactive
100
80
60
40
20
0
1.7
2.2
3.4
4.0
5-1-1
c100-3
c33c
c22-3
RIBA antigen

Figure 4.10. LEGEND

Frequency of reactivities to antigens in the RIBA-2 assay by samples from blood donors infected with different genotypes of HCV. The values above the histogram indicate the mean score for each type with each antigen, from 0 (nonreactive) to 4+. Samples from 252 type 1, 86 type 2, 73 type 3, 19 type 4, 1 type 5, and 12 type 6 infected donors were used for comparison.

Table 4.10. PATTERNS OF SEROLOGICAL REACTIVITY IN CHIRON RIBA-2 ASSOCIATED WITH DIFFERENT GENOTYPES OF HCV

RIBA-2 Pattern						HCV GENOTYPE							
5-1-1	c100-3	c33c	c22-3	Number	PCR+	1	2	3	4	5	6	U[a]	M[b]
RIBA-2 CONFIRMED													
+	+	+	+	271	222	187	15	5	8	0	4	1	2
+	-	+	+	19	12	12	0	0	0	0	0	0	0
-	+	+	+	70	50	10	14	19	3	1	3	0	0
-	-	+	+	166	137	33	56	35	7	0	5	1	0
			Total no.	**526**	**421**	**242**	**85**	**59**	**18**	**1**	**12**	**2**	**2**
RIBA-2 INDETERMINATE													
+	-	-	-	8	0	0	0	0	0	0	0	0	0
-	+	-	-	51	0	0	0	0	0	0	0	0	0
-	-	+	-	24	2	2	0	0	0	0	0	0	0
-	-	-	+	339	24	8	1	14	1	0	0	0	0
+	+	-	-	8	0	0	0	0	0	0	0	0	0
			Total no.	**430**	**26**	**10**	**1**	**14**	**1**	**0**	**0**	**0**	**0**

[a] U : Two samples from Egyptian donors could not be classified by RFLP analysis.
[b] M : Two samples showed evidence of mixed infection with types 1 and 6.

4.4.4. INVESTIGATION OF THE PREVALENCE AND DISTRIBUTION OF HCV TYPE 5.

The study described in section 4.4. examined the distribution of HCV genotypes in blood donors from nine different countries, some genotypes (types 1, 2, and 3) showed a broad distribution while others were detected in one country only (types 4 and 6). Infection with HCV type 5 has been reported to occur in South Africa (Bukh et al., 1992) and has only rarely been found in Europe or elsewhere (section 4.4.3.; McOmish et al., 1994). To further investigate the prevalence and distribution of HCV type 5, blood donor samples from two different areas in South Africa were tested using an RFLP system that detects the six known genotypes of HCV (section 4.3.4.; McOmish et al., 1994).

4.4.5. SAMPLES

Eighty four blood donor plasma samples (42 black donors and 42 caucasian donors) from the Johannesburg area and 29 samples from the Durban area that had been screened by second generation ELISA and found to be repeatedly reactive were shipped frozen to Edinburgh for RT-PCR testing (kindly provided by Dr. Kew, University of Witwatersrand, Johannesburg and Dr. Conradie, The Natal Institute of Immunology, Division of The Natal Blood Transfusion Service, Durban, respectively). PCR positive samples were tested by RIBA-2 by Dr. Brian Dow at Ruchill Hospital. In interpreting the RIBA-2 results plasma with reactivity to both 5-1-1 and c100-3 antigens were considered as indeterminate (see section 4.4.2.).

4.4.6. RESULTS

(1) PCR AND HCV TYPING

Sixty three of the 84 (75%) and 25 out of 29 (86%) samples were PCR positive from Johannesburg and Durban respectively. HCV genotype was determined by RFLP analysis using the restriction endonucleases HaeIII/RsaI and MvaI/HinfI which enables all six known HCV genotypes to be identified (section 4.3.4.; McOmish et al.,1994). Electrophoretic patterns obtained could be assigned to one of the six genotypes in all but 3 of the samples from Johannesburg (table 4.11.). Sequence analysis in the 5′NCR in three of the samples showed that a A→U change at position -177 eliminated the usually well conserved RsaI restriction site producing bands of 217, 9 and 26 bp. All three sequences show similarities to type 4 sequences in the 5′NCR but await sequence analysis in core and NS5 regions to allow classification. In both centres types 1 and 5 were the predominant genotypes found with some infections attributed to types 2, 3, and 4 (table 4.12.). It was not possible to obtain information on the geographical origin of the donors infected with types 3 and 4, however the one donor from Durban that was infected with HCV type 2 had a surname consistent with a Far Eastern ethnic origin.

Table 4.11.

ELECTROPHEROTYPES DETECTED IN DONORS FROM SOUTH AFRICA

	ELECTROPHEROTYPE											
CENTRE	**aA**	**bA**	**cD**	**dD**	**eD**	**fC**	**gC**	**aC***	**bC***	**aD**	**bD**	**hB**
Durban	-	7	1	-	-	3	2	-	-	2	10	-
Johannesburg	2	17	1	-	-	2	-	1	-	12	25	-
Total	**2**	**24**	**2**	**-**	**-**	**5**	**2**	**1**	**-**	**14**	**35**	**-**

Table 4.12.

PREVELANCE OF HCV TYPES IN BLOOD DONORS IN SOUTH AFRICA

CENTRE	**n**	**1**	**2**	**3**	**4**	**5**	**6**
Durban	25	7 (28%)	1 (4%)	5 (20%)	0	12 (48%)	0
Johannesburg	60	19(32%)	1 (1.7%)	2 (3.3%)	1 (1.6%)	37 (62%)	0
Total	**85**	**26(30.1%)**	**2 (2.4%)**	**7 (8.3%)**	**1 (1.2%)**	**49 (58%)**	**0**

* Three donors were infected with variants that could not be classified, bands of 217, 9, and 26 bp were obtained with the HaeIII/RsaI digest and pattern C with the HinfI/MvaI digest.

The distribution of the HCV types was similar in black and caucasian blood donors, of the 26 type 1 sequences identified 12 and 14 were from caucasian and black donors respectively. The 49 HCV type 5 sequences identified were similarly split between 20 caucasian donors and 29 black donors.

(2) SEROLOGY

RIBA-2 results were available on all 25 of the PCR positive samples from Durban and on 46 of the 60 PCR positive samples from Johannesburg (table 4.13.). A similar trend was found as before (section 4.4.3.(2)), those infected with HCV type 1 showed broad reactivities with all four antigens in the RIBA-2 with a high mean score in all cases (fig. 4.11. and table 4.13.). Of the 39 donors infected with HCV type 5 reactivity to the 5-1-1, c100-3, c33c, and c22-3 antigens was 53%, 67%, 75%, and 95% repectively (fig. 4.11. and table 4.13.). The mean score for reactivity to the NS4 encoded antigens was low. Such a response is similar to the serological profile found in individuals infected with HCV types other than type 1. The antibody response produced by the 7 donors infected with HCV type 3 showed reactivity to the c33c and c22-3 antigens only. (HCV types 2 and 4 were represented by 1 sample each in this study, both samples showed reactivity with the c33c and c22-3 antigens only). Of the 18 PCR positive samples that were indeterminate in the RIBA-2, all but one showed reactivity to the c22-3 antigen only. Antibody elicited by one type 5 sample showed reactivity to the c100-3 antigen only which is uncommon in a PCR positive sample.

Figure 4.11.

Frequency of reactivity to antigens in the RIBA-2 by samples from 22 and 39 blood donors infected with HCV type 1 and 5 respectively. The values above the histogram indicate the mean score for each type with each antigen, from 0 (nonreactive) to 4+.

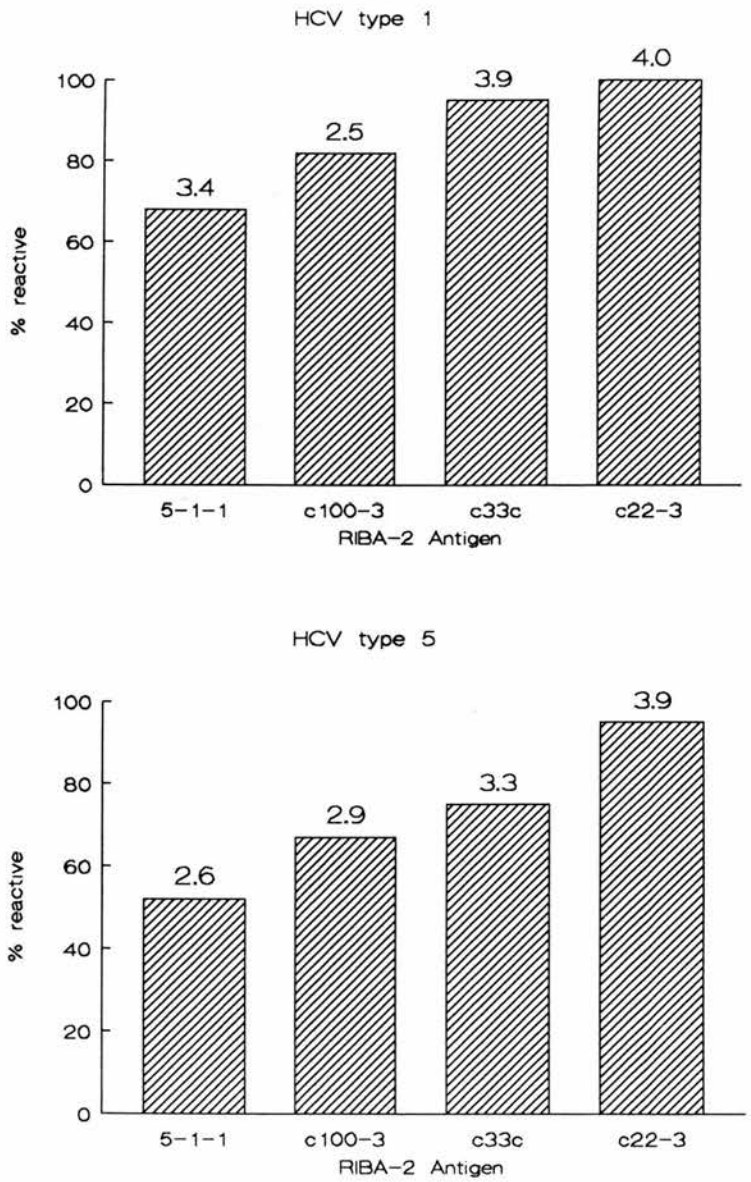


Table 4.13.
PATTERNS OF SEROLOGICAL REACTIVITY IN THE RIBA-2 ASSOCIATED WITH DIFFERENT GENOTYPES OF HCV

RIBA-2 Pattern					HCV GENOTYPE					
5-1-1	c100-3	c33c	c22-3	PCR+	1	2	3	4	5	6
RIBA-2 CONFIRMED										
+	+	+	+	33	14	0	0	1	18	0
+	-	+	+	4	1	0	0	0	3	0
-	+	+	+	6	2	0	0	0	4	0
-	-	+	+	18	4	1	6	0	7	0
			Total no.	**61**	**21**	**1**	**6**	**1**	**32**	**0**
RIBA-2 INDETERMINATE										
+	-	-	-	0	0	0	0	0	0	0
-	+	-	-	1	0	0	0	0	1	0
-	-	+	-	0	0	0	0	0	0	0
-	-	-	+	8	1	0	1	0	6	0
+	+	-	-	0	0	0	0	0	0	0
			Total no.	**9**	**1**	**0**	**1**	**0**	**7**	**0**

Samples not available for testing by RIBA-2 : Type 1 = 3; Type 2 = 1; Type 5 = 10
Three samples not classified by RFLP.

4.4.7. DISCUSSION

The main problem with any HCV typing assay is the need for continual modifications as new HCV genotypes are discovered. Many of the assays that use type-specific primers could be predicted to either fail to differentiate genotypes, or fail to amplify sequences unrecognised when the primers were originally designed. For example, none of the primers used for identification of genotypes I, II, III, and IV (types 1a, 1b, 2a, 2b) described by Okamoto et al., (1990) would be likely to amplify either type 3 and type 4 sequences. Similarly, type-specific probes for amplified NS-5 or 5´NCR based on these genotypes would fail to hybridise or hybridise inappropriately to many of the newly recognised genotypes (Enomoto et al., 1990; Stuyver et al., 1993; Takada et al., 1993).

There is now a considerable amount of comparative sequence data from several geographical regions in the 5´NCR of HCV types 1, 2 and 3. It is therefore possible to predict a comprehensive range of possible electropherotypes with the restriction enzymes used, and to reliably differentiate these genotypes from each other. It is likely that a wider range of electropherotypes will be found for genotypes, and continuous modification of fig.4.8. will be required as new data accumulates. Some of the sequences closely resembling type 4 (Simmonds et al., 1993b,c), but which were obtained from West Africa rather than the Middle East (Bukh et al., 1992; Stuyver et al., 1993) would produce patterns with HaeIII/RsaI other than a and b, although all would produce the C electropherotype with MvaI/HinfI.

The RFLP method described only detects the majority circulating sequence of HCV; co-infection with two or more genotypes is only apparent when approximately equimolar concentrations of different sequences are present within a sample. Only two co-infections (both from Hong Kong donors infected with type 1 and type 6) were detected and could be an underestimate of the true rate of mixed infection. Assays based on type-specific amplification of different genotypes would be expected to be more sensitive indicators of multiple infection (Kato et al., 1991; Okamoto et al., 1993), provided that separate amplification reactions were carried out for each pair of type-specific primers.

This large scale survey of blood donors shows that major differences exist in the geographical distribution of HCV variants. Donors in western European countries (Scotland, Holland and Finland) were almost exclusively infected with types 1, 2 and 3 while those in the Far East (Japan, Taiwan, Hong Kong) were mainly infected with type 1, with lower frequencies of type 2 and type 6 in Hong Kong. The situation is highly complex and difficult to interpret as there are genotypes with world-wide distributions (eg. type 1, 2) co-existing with those which are apparently confined to specific geographical areas (type 4, 5, and 6).

The data from blood donors in this survey is consistent with that obtained from smaller surveys of a variety of different patient groups with non-A, non-B hepatitis or hepatocellular carcinoma (Kato et al., 1991; Takada et al., 1992a,b; Takada et al., 1993). However, these latter studies are potentially biased towards those genotypes

that are more likely to cause more severe liver disease. Current evidence suggests that genotype 1 is associated with a higher rate of chronic active hepatitis or cirrhosis than genotypes 2, 3 or 4 (Dusheiko et al., 1994), and a much poorer response to treatment with α-interferon compared with type 2 (Kanai et al., 1992; Takada et al., 1992b; Yoshioka et al., 1992) and type 3 (Dusheiko et al., 1994). It is possibly significant that amongst the asymptomatic blood donors in this study, a much higher rate of non-type 1 variants (43%) was found than in previous surveys.

More complex distributions of HCV genotypes may be found as more countries are surveyed. HCV type 3 has been found in Thailand and India (pers comm. Dr. L. Jarvis, Dept. Medical Microbiology, Edinburgh). In the former country, type 3 accounts for approximately two thirds of HCV infections in haemophiliacs treated with locally prepared factor VIII concentrate (Jarvis et al., 1994 manuscript in press JID). Type 5 is only commonly found in South Africa (this study, section 4.4.4.; Bukh et al., 1992), although it does occur sporadically elsewhere (one donor identified in Australia in this study and another in Northern Ireland detected when screening blood donations).

Any attempt at interpretation of these geographical differences is hampered by current ignorance surrounding the method of virus transmission. Needle sharing, transfusion of blood and blood products, tattooing and other forms of parenteral exposure have been implicated in the transmission of HCV, while the current evidence suggests that intra-familial, mother-to-child, sexual or inter-familial

transmission are relatively inefficient modes of virus spread. There is therefore little understanding of how or whether HCV might spread in societies where blood transfusion and parenteral drug abuse are uncommon. It is difficult to interpret the data obtained, similarity in distribution of HCV genotypes in Australia compared with western Europe (and its difference from other Far East countries) could be explained by assuming an ancient origin for HCV, and relating the distribution of genotypes to the original European ancestry of the majority of Australian blood donors. On the other hand, it may be that HCV was relatively recently introduced by importation of contaminated commercial blood products manufactured from paid donations collected in North and Central America, or by needle sharing with HCV-infected individuals from Europe or the USA.

The existence of distinct HCV genotypes with up to 30% sequence diversity could potentially result in significant antigenic variation among homologous proteins encoded by the different genotypes of HCV. The effect that this sequence variation would have on the effectiveness of antibody screening for HCV has not been widely studied. All current assays use antigens derived from HCV type 1 sequences, and rely on cross-reacting antibody to detect infection with other HCV genotypes. This is illustrated by the first generation assays that used the c100-3 recombinant protein derived from NS-4. The antigenic region was found to be particularly variable in sequence between HCV types 1, 2 and 3 (Simmonds et al., 1993a), and led to a low rate of detection of infection with variants other than type 1 (Chan et al., 1991; McOmish et al., 1993a). In this study, the majority of individuals infected with

genotypes 2 to 6 show weak or absent reactivity to c100-3, confirming similar observations for types 2 and 3 on a relatively smaller number of individuals (McOmish et al., 1993a). This lack of cross-reactivity provides at least one explanation for the continued transmission of HCV by blood screened by anti-C100 assays alone (Esteban et al.,1990; Japanese Red Cross NANBH Research Group, 1991).

All donors infected with types 2 to 6 reacted with the recombinant core protein, c22-3 reflecting its greater degree of amino acid sequence conservation (90%) than the non-structural proteins (75-80%). However, this observation is misleading because c22-3 is a component of screening and confirmatory assays for HCV infection. In interpreting these results, the pre-selection by the screening assays precluded an analysis of the proportion of infections with divergent HCV types that elicited an even more restricted serological response so as to be undetectable by current blood donor screening. The actual frequency with which samples from individuals infected with variants other than type 1 are missed by current 2nd generation screening assays (that contain c33c and c22-3 as well as NS-4 proteins) is unknown, but probably low. However, it is recognised that the serological response to HCV-encoded antigens is often narrow in specificity, and is generally of low titre. Acute infection with HCV following transfusion of infectious blood or blood products may fail to elicit antibody for several months (van der Poel et al.,1992). Furthermore, individuals who are only marginally immunosuppressed (such as renal dialysis patients, the elderly, haemophiliacs and neonates) generally show extremely restricted and idiosyncratic

patterns of serological reactivity, often to only one (or possibly none) of the four antigens used in current screening assays (Lelie et al., 1992). In all of these cases, it would be beneficial to have a test that is optimally sensitive for all variants of HCV. Evaluation of assays that incorporate additional peptide antigens corresponding to epitopes of other HCV types not shared with type 1 may represent a first step in improving current assays.

4.5. COMPARISON AND VALIDATION OF A TYPE SPECIFIC ANTIBODY ASSAY WITH AN RFLP ASSAY

4.5.1. INTRODUCTION

The NS-4 region encoding the c100-3 antigen is variable between different isolates of HCV, showing only 75-77% sequence similarity between HCV types 1 and 2. There is evidence that this affects serological recognition demonstrated by the relative infrequent serological reactivity of sera from blood donors infected with HCV types 2 and 3 with NS-4-derived antigens in immunoblot assays (section 4.2., McOmish et al., 1993a; McOmish et al., 1994), and frequent negative results in the original 1st generation blood donor screening assays. An investigation of the distribution of epitopes in the NS-4 region encoding c100-3 for HCV types 1, 2 and 3 was carried out (Simmonds et al., 1993a). The frequency of serological reactivity to synthetic peptides corresponding to two antigenic regions of the sequenced region of the three HCV types was examined using samples from blood donors infected with known HCV types determined by RFLP. This enabled an investigation of the frequency of type-specific and cross-reactive antibody specificities to be determined in naturally

infected individuals. The newly developed assay for type-specific antibody was used to detect infection with different HCV types in anti-HCV positive blood donors, and the incidence of mixed infection in haemophiliacs multiply exposed to HCV and compared with the genotype determined by RFLP analysis.

4.5.2. SAMPLES

Plasma samples were obtained from 137 HCV-infected blood donors identified by screening with Abbott or Ortho second generation ELISA. Plasma samples were also obtained from 27 HIV-uninfected haemophiliacs treated with non-heat inactivated factor VIII and IX concentrates and who had been infected with HCV for several years. All samples were anti-HCV positive by several second generation screening and confirmatory assays (Dow et al., 1993; section 4.2.3.). Control samples were obtained from anti-HCV negative blood donors. All samples from anti-HCV positive blood donors were screened for antibody to the recombinant c100-3 recombinant protein using the Abbott 1st generation EIA, according to the manufacturer's instructions.

Development of the type-specific antibody assay and serological typing of samples was carried out by Ken Rose and is described in Simmonds et al, 1993. RFLP analysis was carried out using the combination of enzymes HaeIII/RsaI and ScrFI (section 4.3.2.)

4.5.3. RESULTS

A total of 137 samples from donors typed by RFLP analysis of sequences amplified by PCR in the 5´NCR were assayed blind for the presence of type-specific antibodies using the HCV-TSAA (table 4.14.). Upon de-coding the results, it was found that there was good correlation between the results of the two assays. Almost all samples that could be serologically typed contained antibody of a single type-specificity corresponding to the HCV type detected by PCR (118/122). Antibody to one of the HCV types found in the four samples with dual specificity also corresponded to the circulating HCV type identified by PCR. Only 15 samples could not be typed using the serological assay; 7 were non-reactive with the NS-4 antigens at either 1/40 or upon retesting at a 1/10 test dilution, while the remaining 8 contained no detectable type-specific antibody.

To investigate whether the HCV-TSAA could reliably detect multiple infection, samples were tested from 27 HCV-infected haemophiliacs treated for several years with non-heat treated factor VIII or IX, and who have potentially been exposed to all three types of HCV over the years of treatment (Ludlam et al., 1989; Watson et al., 1992). It was found that a higher proportion of samples showed type-specific antibody to multiple HCV types. In blood donors, only 4 samples out of 122 showed evidence of multiple infection (3%), dual or even triple antibody specificities were found in 8 of the 20 haemophiliac samples typed by HCV-TSAA (40%; table 4.14.). Antibody to types 1 and 3 was most frequently found in the haemophiliacs. Most had only been treated with factor VIII or IX concentrates derived from local plasma

Table 4.14.

COMPARISON OF SEROLOGICAL TYPING BY HCV-TSAA WITH PCR

	Number	TYPE-SPECIFIC ANTIBODY								
PCR[a]	tested	1	2	3	1+2	1+3	2+3	1+2+3	NTS[b]	NR[c]
1	68	61	-	-	-	1	-	-	3	3
2	13	-	11	-	-	-	1	-	1	0
3	56	1	-	45	-	2	-	-	4	4
Hem[d]	27	10	-	2	1	6	-	1	2	5

[a] Genotype of HCV sequences amplified by PCR and typed by RFLP

[b] NTS : No type-specific antibody detected

[c] NR : non-reactive with NS-4 peptides

[d] Samples from HCV-infected haemophiliacs, un-typed by PCR.

donations, and is consistent with previous observations these two types are the predominant HCV variants detected in Scottish blood donors (section 4.3.3. and McOmish et al., 1993a).

4.5.4. DISCUSSION

The amino acid sequences of the antigenic regions of the three HCV types differed considerably from each other. In the attempt to develop a type-specific antibody assay (TSAA) it was assumed that serological reactivity to them would be type-specific. However, substantial degrees of cross-reactivity were observed upon epitope mapping and ELISA with branched peptides (Simmonds et al., 1993a). To detect type-specific antibody, it was necessary to absorb out cross-reactive antibody with a molar excess of heterologous peptides in solution, on the basis that the only antibody that could bind to the solid phase was that which reacted with epitopes not shared with other HCV types. Using this modification to the assay, in general, the circulating HCV genotype detected by PCR/RFLP in samples from HCV-infected blood donors corresponded to the detection of type-specific antibody to the corresponding HCV serotype (table 4.14.). The detection of dual antibody specificity in samples from four donors suggested that they had been exposed and infected with more than one HCV type; in such cases the PCR type corresponded to one of the HCV types for which type-specific antibody was detected. A total of 122 of 137 blood donor samples were successfully typed (89%) serologically, of which all but one were consistent with HCV genotyping by PCR. Although the serological assay for type-specific antibody to different HCV types does not necessarily reflect actively

replicating virus in cases of recent infection, it is possible that this could provide a practical alternative to the PCR-based typing assays (Enomoto et al.,1990; McOmish et al., 1993a; Nakao et al., 1991; Okamoto et al., 1991) currently available, and the only possible assay in those who are anti-HCV positive, but PCR negative.

In this study it was found that almost all blood donors had antibody to only a single type. This is perhaps surprising in view of previous observations that the major risk factor for HCV infection in this particular group of blood donors is past intravenous drug abuse by needlesharing and that both HCV type 1 and 3 are approximately equally prevalent in this particular risk group in Scotland (section 4.3.3.; McOmish et al., 1993; Crawford et al., 1994). A higher rate of multiple infection was detected in haemophiliacs treated with non-inactivated factor VIII or IX (40%), higher than that detected by PCR-based typing of circulating RNA sequences (Okamoto et al., 1992b), but which still does not reflect the full extent of their exposure to HCV over the years of treatment with factor VIII. It is possible that past infection with HCV completely or partially protects the individual from further infection, or attenuates the secondary immune response to the extent that type-specific antibody to the re-infecting virus type remains undetectable.

CHAPTER 5

5. HEPATITIS C VIRUS TRANSMISSION BY INTRAVENOUS IMMUNOGLOBULIN

5.1. GENERAL INTRODUCTION

Until recently intravenous immunoglobulin (IVIG) preparations had a very good safety record with respect to transmission of viruses. Even in the absence of specific virus inactivation procedures, immunoglobulins prepared by the Cohn procedure have been free of disease transmission. In this process, serum proteins are separated by a sequence of precipitations with ethanol at low temperature, ionic strength and pH. Contaminating viruses partition into the precipitated fractions which are discarded in the production procedure (Louie et al., 1994). Immunoglobulin G constitutes 95 to 99 percent of the protein in Cohn Fraction II with varying small quantities of IgA, IgM, IgD, IgE and other proteins. In the final stages of production contaminating ethanol is removed under conditions designed to prevent the formation of aggregated immunoglobulin, while further processing (finishing steps) reduces the levels of spontaneous anticomplementary activity and vasoactive substances. Finishing steps used by different manufacturers are DEAE sephadex filtration or treatment at pH4.0 with low concentrations of pepsin, the latter treatment providing a degree of virus inactivation (Louie et al., 1994; pers comm Dr.H.Hart, Protein Fractionation Centre, Edinburgh).

The following sections describe and investigate HCV transmission by two different immunoglobulin preparations. The first, from normal human immunoglobulin used in the treatment of immunodeficient patients and the second, from a specific

immunoglobulin (anti-D immunoglobulin) administered to RhD- women after pregnancy. Different manufacturing processes were used in the preparation of these different batches of immunoglobulin and is relevant in considering how HCV transmission by these products may have occurred.

5.2. A RETROSPECTIVE STUDY OF IMMUNODEFICIENT PATIENTS

5.2.1. INTRODUCTION

Intravenous immunoglobulin (IVIG) preparations allow large amounts of immunoglobulin to be administered rapidly and painlessly to patients with deficiencies in antibody production. Individuals with primary immunodeficiency diseases such as X-linked agammaglobulinemia (XLA), common variable immunodeficiency (CVI) and X-linked immunodeficiency with hyperimmunoglobulinemia M are characterised by low or undetectable amounts of all five serum immunoglobulin classes. Another group of patients who benefit from IVIG administration are those with normal or near-normal immunoglobulin levels but who have marked impairment in their ability to produce specific antibodies after immunization. Such patients include males with the Wiskott-Aldrich syndrome who are unable to produce antibodies to polysaccharide antigens and have blunted anamnestic responses to protein antigens, and patients with ataxia telangiectasia, a hereditary degenerative disease of the spinal cord, cerebellum and other parts of the nervous system. Children with transient hypogammaglobulinemia of infancy may have low serum concentrations of all or some immunoglobulins during the first few months of life, although normal amounts of specific antibodies in response to

immunization are often observed. The administration of exogenous antibody may inhibit endogenous antibody formation in these patients (Tiller et al., 1978). The only clear indication for replacement therapy with intravenous immunoglobulin in patients with selective IgG subclass deficiency is a demonstrated deficiency in the ability to form antibodies against a variety of polysaccharide and protein antigens.

Secondary immunodeficiency may be the result of disease or trauma (Berkman et al., 1990). Haematological cancer patients or individuals receiving immunosuppressive agents for the treatment of cancer may be antibody deficient. Those with HIV infection, particularly infants, may have defects in their ability to form specific antibodies, even though serum immunoglobulin levels are usually elevated. Administration of intravenous immunoglobulin to HIV infected individuals may prolong survival by reducing the risk of opportunistic infection.

IVIG produced by Cohn fractionation using mild manufacturing methods in the finishing step, such as gel chromatography and ultrafiltration (or none at all), have been implicated in the transmission of NANBH (Lane, 1983; Lever et al., 1984; Ochs et al., 1985; Ochs et al., 1986; Weiland et al., 1986; Hammarstrom and Smith, 1986; Bjorkander et al., 1988; Quinti et al., 1990), and also from one product treated at pH4 with low concentrations of pepsin in the final stage (Williams et al., 1989). At the time of publication of these reports the agent of NANBH was yet to be confirmed as the hepatitis C virus (HCV) and reliable serological assays were not readily available. As a result the diagnosis of NANBH was generally one of exclusion (of

HBV and CMV) in the presence of abnormal liver function tests or histological appearance in liver biopsies. In addition most of the patients involved suffered from either X-linked agammaglobulinaemia (XLA) or common variable immunodeficiency (CVI) (Lever et al., 1984; Ochs et al., 1985; Ochs et al., 1986; Weiland et al., 1986; Hammarstrom and Smith, 1986; Bjorkander et al., 1988; Williams et al., 1989; Quinti et al., 1990) and therefore were unable to mount an antibody response. Direct detection of virus nucleic acid would be the definitive method of diagnosis.

Plasma or serum samples from many of the patients reported in these transmission events had been stored at -20°C or colder and were made available for HCV RNA detection using RT-PCR. The original IVIG batches possibly associated with the NANBH transmission were also tested in order to establish the nature of the contaminating infective agent.

5.2.2. SAMPLES

Plasma samples from 33 patients who had developed NANBH (as defined either by symptoms of hepatitis and/or an elevation of liver enzyme levels indicative of a viral hepatitis infection) after receiving one of four different IVIG preparations from different manufacturers (preparations A - D, table 5.1.). Another group of 18 patients had received implicated, or suspected IVIG batches but had normal liver enzymes and no symptoms of hepatitis. In 25 of the patients with IVIG associated NANBH, samples were available prior to the administration of the implicated or suspect IVIG batches. Due to the study being carried out retrospectively, it was possible to obtain

Table 5.1.
INTRAVENOUS IMMUNOGLOBULIN PREPARATIONS TESTED

Product	Manufacturer	Finishing Step
A:BPL	Blood Products Laboratory	Gel Filtration
B:Gammonativ	Kabi Vitrum Sweden	DEAE Sephadex and albumin
C:Gammagard	Hyland Travenol USA	PEG, DEAE Sephadex
D:SNBTS IVIG	Scottish National Blood Transfusion Service	pH4 and pepsin

Note : All preparations used cold ethanol fractionation (Cohn Method) as the primary manufacturing procedure. The finishing step is the final stage of production.

samples that had been collected at varying periods before and after exposure to the IVIG preparations. All samples were received coded and tested blind with the code being broken when results were available.

Patient details are described and summarised in table 5.2..

(i) Five patients (2 XLA, 2 CVI and 1 hyper IgM syndrome; cases 1-5). All developed NANBH after receiving pilot batches of experimental IVIG preparation A in 1982 (Lever et al., 1983).

(ii) Five patients with CVI (cases 6-9) who developed NANBH in 1985 after therapy with IVIG preparation B. For this group it was not possible to identify specific IVIG batches associated with NANBH transmission, however these patients could be compared with samples from six other patients with CVI (cases 10-15) who were treated with IVIG batches manufactured at the same time but who did not appear to develop NANBH.

(iii) Ten patients (8 CVI and 2 IgG subclass deficiency; cases 16-21 and 23-26) who developed NANBH after therapy with IVIG preparation B in 1983-1985. Four of these patients (cases 23-26) had previously been treated in 1978-1979 with an experimental IVIG preparation manufactured in Sweden (Bjorkander, unpublished data). One patient (case 22) was treated with IVIG preparation B at the same time but did not develop NANBH.

(iv) Ten patients (3 possible XLA and 7 CVI; cases 27-36) who developed NANBH following exposure to two pilot batches of experimental IVIG preparation C in 1983 were compared with seven patients (1 possible XLA, 1 X-linked immunodeficiency with hyper-IgM and 5 CVI; cases 37-43) who did not develop NANBH despite exposure to the same two experimental batches of the IVIG preparation. An additional patient (case 28) received a single transfusion of the contaminated IVIG batch, and increase in ALT values was observed and the patient developed the symptoms of chronic active hepatitis. Another patient (cases 43) died in 1983 four months after receiving the contaminated batch of IVIG. Although the ALT values remained normal for this patient, chicken pox and idiopathic thrombocytopenia were thought to be complicating factors.

(v) Four patients (2 XLA and 2 CVI; cases 44-47) who developed NANBH after treatment with a single batch of IVIG preparation D in 1988. Liver tissue stored at -20^{0}C after necropsy was available from one of the patients with NANBH (case 44). Samples were also available from 4 patients (3 CVI and 1 with drug induced hypogammaglobulinaemia; cases 48-51) who were treated with the implicated IVIG batch D, but did not develop NANBH.

5.2.3. INTRAVENOUS IMMUNOGLOBULIN PREPARATIONS

Only three of four implicated preparations of IVIG were available for testing for the presence of HCV RNA. Fourteen batches of IVIG preparation B, possibly associated with NANBH transmission, and two batches of IVIG preparation C, definitely

implicated in NANBH transmission, were stored as a freeze dried powder at 4^0C prior to reconstitution according to the manufacturers instructions immediately before RNA extraction. One batch of IVIG preparation D, definitely associated with NANBH transmission had previously been reconstituted as a 50g/l solution after manufacture and stored at -20^0C prior to being used in this study, together with 9 other IVIG batches manufactured and stored in the same way.

5.2.4. RESULTS

Samples were available for testing for the presence of HCV RNA by PCR from 33 patients who had developed hepatitis after exposure to one of four different IVIG preparations (table 5.2.). For 24 (73%) of these patients, samples were available that had been taken prior to therapy with IVIG batches implicated or suspected of transmitting NANBH, 7 (29%) were found to be HCV positive by PCR prior to IVIG administration. This suggests that they had already been infected with HCV by a source other than the implicated or suspected batches of IVIG.

Fifteen out of 17 patients, who were initially negative for HCV RNA, were found to be positive after IVIG therapy and all 15 developed NANBH. Eight of 9 patients (89%) for whom a sample was not available prior to IVIG therapy were also found to be positive for HCV RNA after IVIG treatment. This implies that HCV is the virus that is most likely to be responsible for the NANBH in these two groups of patients. HCV RNA could also be detected in liver tissue from one patient who had received IVIG preparation D and subsequently developed NANBH. Six patients who

Table 5.2. PATIENT DETAILS

Prep	Patient case no.	Control case no.	HCV PCR +/- Pre IVIG months(m) and years(y)	Post IVIG
A	1			+(7y)
	2			+(7y)
	3		-(9m)	+(1y)
	4			+(1y)
	5			+(3y,4y)
B	6			+(2y)
	7		-(1m)	+(1m)
	8			+(4y)
	9			+(4y)
		10		-(2y)
		11		-(2y)
		12		-(1y)
		13		-(1y)
		14		-(4y)
		15		-(2y)
	16		-(1m)	+(7m)
	17		-(1m)	+(6m)
	18		-(1y)	+(3y)
	19		-(1m)	+(10m)
	20		-(1m)	-(4m)
	21		+(1m)	-(4m)
		22		-(4y,5y)
	23		+(1m)	+(6m)
	24		+(1m)	+(6m)
	25		+(2y)	+(2y,2y6m)
	26		+(1m)	+(1y,5y,5y4m)
C	27		-(5y,1m)	+(4y,5y,7y)
	28		-(4y,2y)	+(4m)
	29		-(2y)	+(15m)
	30		-(3m)	+(1m,2y,5y)
	31		-(10m)	+(2m,15m,5y)
	32		-(7m,1m)	+(4y,5y,6y)
	33		-(3y)	-(17m),+(6y)
	34		-(2y)	-(14m)
	35		+(5m)	+(4m,8y)
	36		+(5y,5m)	+(1m)
		37	-(3y,1m)	-(3y,6y)
		38	-(2y)	-(3m,10y)
		39	-(14y)	-(16m,8y)
		40	-(1y)	+(5y)
		41	-(1y)	+(2m)
		42	-(1y)	+(1y,8y)
		43	-(1y,3m)	+(4m)

Table 5.2. continued

Prep	Patient case no.	Control case no.	HCV PCR +/- Pre IVIG months(m) and years(y)	Post IVIG
D	44		-(2m)	+(2m,2y)
	45		-(5m)	+(3m)
	46			+(1y)
	47			-(1y)
		48		-(5m)
		49		-(5m)
		50		-(5m)
		51		-(6m)

were negative for HCV RNA prior to IVIG therapy had more than one sample available after the development of NANBH. Five of these samples were repeatedly positive (with an interval of up to eight years) suggesting that once HCV infection occurs it persists (in this patient category). Another group of eighteen patients had received implicated or suspected IVIG batches but had not developed NANBH. Samples were available from 7 of these patients prior to the administration of implicated or suspected IVIG batches; 2 patients were positive for HCV RNA before IVIG therapy (cases 42 and 43). Four of the 7 were HCV RNA negative after IVIG therapy but three patients became HCV RNA positive despite the absence of symptoms of NANBH. Of the eleven patients for which there was no sample for RT-PCR analysis prior to IVIG therapy, all were found to be negative for HCV RNA in the post-treatment sample.

Eight out of 9 patients who were positive for HCV RNA prior to receiving an implicated or suspected batch of IVIG, remained positive over a period of up to 8 years (except case 21). This is a similar trend to that found with the patients who were negative for HCV RNA prior to IVIG therapy, supporting the hypothesis that once infected by HCV, immunodeficient patients remain so. Four patients (cases 23-26) were found to be positive for HCV RNA. At first the route of exposure was not clear (these patient should have been in a group that had no previous IVIG treatment), on investigation it was found that they had been exposed to an experimental IVIG batch in 1978-1979 which was manufactured in Sweden (Bjorkander, unpublished data). Samples from these four patients prior to 1979 were

retrieved from storage and found to be negative for HCV RNA (data not shown). Five patients were positive for HCV RNA (cases 21, 35, 36, 42 and 43) in which a definite source of exposure to HCV could not be identified. However, 1 patient (case 35) had blood transfusions related to surgery; 1 (case 36) had symptoms of hepatitis 10 years earlier, 1 (case 42) was a Vietnam Veteran and 1 (case 43) had received multiple red blood cell and platelet transfusions during the preceding year.

HCV RNA could not be detected in four patients who had NANBH after IVIG therapy (cases 20, 33, 34 and 47). One of these patients (case 47) was subsequently found to have had a spontaneous improvement in plasma ALT levels, and so may no longer have been viraemic.

Of the 3 (out of 4) different IVIG preparations available, 2 batches of preparation C implicated in NANBH and 6 out of 14 batches of preparation B which were strongly suspected of transmitting NANBH, had detectable levels of HCV RNA. The implicated batch of IVIG preparation D, and another 9 batches of the same preparation manufactured at the same time but not implicated in NANBH transmission, were found to negative for HCV RNA.

5.2.5. DISCUSSION

A retrospective study was carried out to identify the agent responsible for causing NANBH in a group of patients who had been treated with IVIG. The use of RT-PCR has been shown to be effective for detecting HCV RNA in plasma, serum, factor

concentrate and IVIG preparations that have been stored over a number of years (Simmonds et al., 1990; Garson et al., 1990). The 5′ NCR primers used are capable of detecting the presence of all currently known HCV types (chapter 4, section 4.4.). Serum or plasma samples that had been collected prior to the development of NANBH were also tested. A separate group were patients who had been exposed to IVIG batches which were implicated (or strongly suspected) of causing NANBH transmission, but these patients had not developed NANBH.

Thirty three patients who had developed NANBH associated with the administration of IVIG were studied. However, 7 of these patients (and two patients, cases 42 and 43, who had no symptoms of hepatitis) were positive for HCV RNA prior to exposure to IVIG, implying that the source of infection was other than the implicated or suspected IVIG batches. Four of these patients (cases 23-26) were exposed to an experimental IVIG preparation in 1978-1979, 2 patients had previously been transfused with whole blood and another had a history of hepatitis and had served in the Vietnam War. Of the remaining patients, 17 were negative for HCV RNA prior to exposure to IVIG batches associated with NANBH and of these HCV RNA could be detected in 15 patients post-exposure. In the group where no sample was available prior to the development of NANBH, 8/9 patients were positive for HCV RNA, suggesting that HCV is the causative agent for the NANBH associated with IVIG administration.

Testing the implicated batches of IVIG showed that 6 batches of IVIG preparation B and 2 batches of IVIG preparation C contained HCV RNA. The batches of IVIG preparation B were routine production batches by the Cohn fractionation method with albumin stabiliser added and DEAE-purification as the finishing step. In a prospective study over twelve months of follow-up of immunodeficient recipients receiving monthly infusions of IVIG preparation B, there was no evidence of NANBH transmission (Bjorkander et al., 1991) for this preparation in which a solvent-detergent virucidal treatment step had been incorporated into the manufacturing procedure. The batches of IVIG preparation C were produced in a pilot plant. A study on liver enzyme levels in patients who had received the FDA licensed version produced in a large scale manufacturing facility, did not indicate that any transmission of NANBH (Lee et al. 1988). HCV RNA was not detected in IVIG preparation D and a follow up of immunodeficient recipients of subsequent batches of this preparation over a 2 year period did not find any further cases of NANBH (pers comm Dr.Yap, Scottish National Blood Transfusion Service, Edinburgh).

Studies assessing the safety of IVIG preparations have been difficult due to the sporadic nature of transmission episodes, the lack of a serological response by immunodeficient patients with IVIG associated NANBH and the small number of cases available for study. The patient group used in this study is of a reasonable size, their cases are well documented and the causative agent has been identified by detection of virus nucleic acid.

5.3. HCV TRANSMISSION BY ANTI-D IMMUNOGLOBULIN

5.3.1. INTRODUCTION

The membranes of human red cells contain a variety of antigens and can be divided into four major blood types; A, B, AB, and O based on the presence of the A and B antigens on the red blood cells (RBCs). Antibodies against these antigens can be produced by exposure to the red cells of another individual. Exposure is most likely to occur via blood transfusion, or during pregnancy when foetal red cells cross the placenta and enter the circulation of the mother. The A and B antigens and those of the Rhesus (Rh) system are of greatest clinical importance. The Rh system is made up of many antigens, the most antigenic being the D antigen. The term RhD+ denotes a person who has the D antigen on their RBCs while a RhD- individual has no D antigen. Eighty-five percent of the UK population are RhD+, with the remainder RhD-. The Rh factor is especially important in pregnancy when a mother with a RhD- blood group can be sensitized by RBCs from a RhD+ baby. This occurs most often at the birth of the first child when bleeding of the placenta can release a large number of the baby's RBCs into the mother. The mother produces antibodies which are predominantly of the IgG class and will be able to cross the placenta in any subsequent pregnancy. Reaction of maternal antibodies with the D-antigen on the RBCs of a second RhD+ baby leads to cell lysis. The resulting haemolytic disease of the newborn (HDN) can vary in severity from mild anaemia to death in utero. Prophylactic treatment with small amounts of IgG anti-D at the time of birth of the first child greatly reduces the risk of sensitization. This is effective because if the mother has circulating IgG anti-D, sensitization to the D antigen is less likely due to

lysis of the foetal RBCs before they encounter antigen sensitive cells and trigger an immune resonse.

Anti-D immunoglobulin is prepared from plasma containing high levels of anti Rh D antibody. Such plasma is obtained by hyperimmunising RhD- volunteers with RhD+ RBCs and anti-D IVIG is usually manufactured by Cohn fractionation as outlined in section 5.1.. The risk of transmission of viruses by anti-D IVIG preparations is low due to the manufacturing methods used and because the products are manufactured from small pools of donors which reduces the chance of introducing viraemic plasma into the pool. There has only been one previous reported outbreak of NANBH from anti-D IVIG which occurred in East Germany following the administration of anti-D IVIG manufactured in 1978 (Dittman et al., 1991). This preparation had not been prepared by Cohn fractionation and the only purification step carried out on the plasma was passage through a DEAE sephadex column. Retrospective testing of sera taken 6-12 months after the administration of the anti-D IVIG confirmed HCV as the infecting agent. Ten years later, 60% of these women have developed chronic hepatitis, and 82.7% of these are anti-HCV positive.

The Blood Transfusion Service Board in Eire introduced routine testing of blood donors for anti-HCV in 1991, in line with procedures in the UK (chapter 4, section 4.2.). A disproportionate number of anti-HCV positive donors in Eire were found to be RhD- female donors, who had children aged 17. On tracing donor records of these individuals most were found to have been administered anti-D immunoglobulin in

1977. A national initiative has recently been undertaken to recall and offer HCV testing to all women who have received anti-D IVIG since its introduction in 1970. The following section describes the results of the first three months of this screen, and provides evidence for a phylogenetic link between the infected batch of IVIG, the original donor and the anti-D IVIG recipients.

5.3.2. SAMPLES

Sixty thousand samples were screened for anti-HCV at centres in Dublin and Cork (by Mr.T.Finch and Mr.J.Willis and staff respectively). All samples repeatedly reactive in the 2nd generation ELISA screening assay (Abbott GmBH, Wiesbade-Delkenhein, Germany) were tested by 3rd generation RIBA (RIBA-3, Chiron Corporation, Emeryville, California). Eight hundred and ninety-seven samples that were positive or indeterminate (chapter 4, section 4.2.) in the RIBA-3 were tested for virus by RT-PCR. Dr.L.Jarvis and J.Daub assisted in the extraction and RT-PCR of these samples.

Four batches of anti-D IVIG were available for testing and a plasma sample from the HCV positive donor whose plasma was present in the pool from which implicated anti-D IVIG batches were manufactured.

5.3.3. RESULTS

Of the 897 samples tested by RIBA-3, 577 (64%) were positive and 320 (36%) indeterminate. Of the RIBA-3 positive samples, 371/577 (64%) were RT-PCR positive compared to 15/320 (4.7%) of the RIBA-3 indeterminate samples. HCV RNA could be detected in all four of the batches of anti-D IVIG tested using primers specific for the 5'NCR.

HCV genotype was determined by RFLP analysis (chapter 4, section 4.3.4.) for 100 RT-PCR positive samples of recipients of anti-D IVIG and all were genotype 1. In contrast, 11 blood donor samples that had risk factors other than anti-D IVIG administration (table 5.3.), infection with genotypes 1, 2 and 3 was detected in 4, 3, and 4 donors respectively demonstrating that HCV types 1, 2 and 3 are present in the donating population in Eire.

To establish that the HCV type 1 infection in the anti-D recipients were related, direct nucleotide sequence analysis of the NS-5 region of the HCV genome was carried out. Single molecules for each sample were obtained by titration to limiting dilution. HCV nucleotide sequences could be amplified in the NS-5 region for sera from 10 anti-D IVIG recipients, 6 blood donors with other risk factors, the implicated batch of anti-D IVIG and from sera from the original HCV infected donor. These sequences were compared with representative 1a, 1b and 2b sequences (fig.5.1.). Their relationship with published NS-5 sequences was investigated by phylogenetic analysis, the results of which are presented in a rooted phylogenetic tree (fig.5.2.).

The sequences from the non anti-D recipient blood donors infected with HCV type 2 (cork B and cork 11), showed similarities to subtype 2b. HCV sequences obtained from 4 non anti-D recipient blood donors infected with type 1 grouped closely to other published sequences within subtype 1a (cork 1, 5, 13 and A). All 10 anti-D IgG recipients (cork 2, 3, 4, 6, 9, 12, 14, 15, 16, and 17), the anti-D IVIG (cork B250) and the implicated donor (labelled : DONOR) grouped with the subtype 1b group of published sequences (fig.5.1. and 5.2.).

Table 5.3.

RIBA-2/PCR POSITIVE SAMPLES AND ASSOCIATED RISK FACTORS

HCV Genotype	No. of Samples	Risk Factor
1	2	IVDA*
1	1	Sexual partner IVDA
1	1	Not known
2	1	Blood Transfusion
2	2	Not known
3	3	IVDA
3	1	Blood Transfusion
1	100	Anti-D IgG

* IVDA : Intravenous Drug Abuse

Figure 5.1.

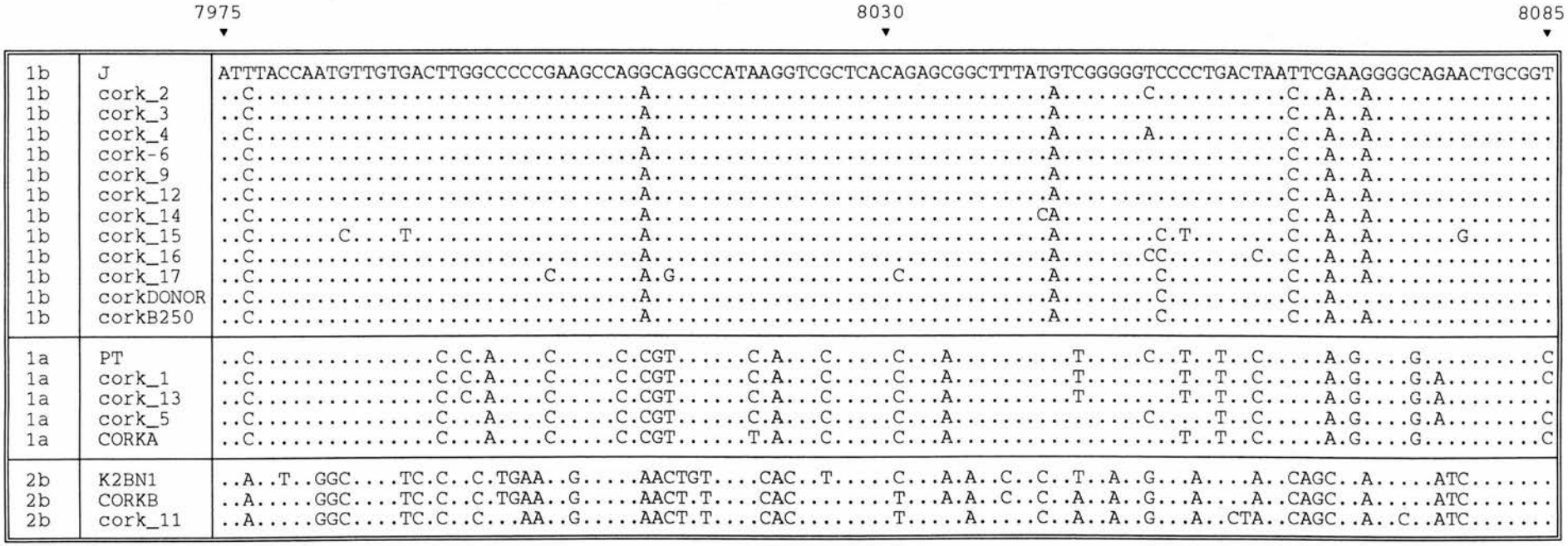

7975
8030
8085
1b J ATTTACCAATGTTGTGACTTGGCCCCCGAAGCCAGGCAGGCCATAAGGTCGCTCACAGAGCGGCTTTATGTCGGGGGTCCCCTGACTAATTCGAAGGGGCAGAACTGCGGT
1b cork_2 ..C.....A.....A.....C.....C..A..A.....
1b cork_3 ..C.....A.....A.....C..A..A.....
1b cork_4 ..C.....A.....A.....A.....C..A..A.....
1b cork-6 ..C.....A.....A.....C..A..A.....
1b cork_9 ..C.....A.....A.....C..A..A.....
1b cork_12 ..C.....A.....A.....C..A..A.....
1b cork_14 ..C.....A.....CA.....C..A..A.....
1b cork_15 ..C.....C....T.....A.....A.....C.T.....C..A..A.....G.....
1b cork_16 ..C.....A.....A.....CC.....C..C..A..A.....
1b cork_17 ..C.....C.....A.G.....C.....A.....C.....C..A..A.....
1b corkDONOR ..C.....A.....A.....C.....C..A.....
1b corkB250 ..C.....A.....A.....C.....C..A..A.....
1a PT ..C.....C.C.A....C.....C.CGT.....C.A...C.....C...A.....T.....C..T..T..C.....A.G....G.....C
1a cork_1 ..C.....C.C.A....C.....C.CGT.....C.A...C.....C...A.....T.....T..T..C.....A.G....G.A.....C
1a cork_13 ..C.....C.C.A....C.....C.CGT.....C.A...C.....C...A.....T.....T..T..C.....A.G....G.A.....
1a cork_5 ..C.....C...A....C.....C.CGT.....C.A...C.....C...A.....C.....T..C.....A.G....G.A.....C
1a CORKA ..C.....C...A....C.....C.CGT.....T.A...C.....C...A.....T..T..C.....A.G....G.....C
2b K2BN1 ..A..T..GGC....TC.C..C.TGAA..G.....AACTGT....CAC..T.....C...A.A..C..C..T..A..G...A....A..CAGC..A.....ATC.....
2b CORKB ..A.....GGC....TC.C..C.TGAA..G.....AACT.T....CAC.....T...A.A..C..C..A..A..G...A....A..CAGC..A.....ATC.....
2b cork_11 ..A.....GGC....TC.C..C...AA..G.....AACT.T....CAC.....T.....A.....C..A..A..G...A..CTA..CAGC..A..C..ATC.....

Figure 5.1. continued

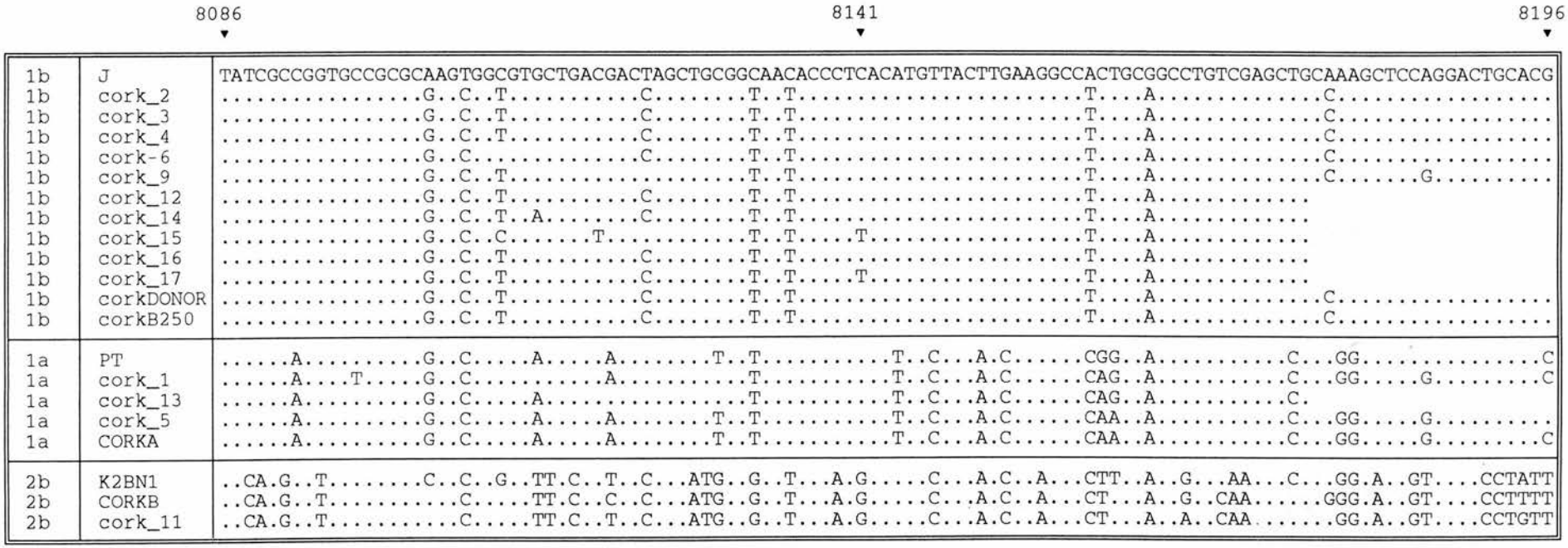


Figure 5.1. LEGEND

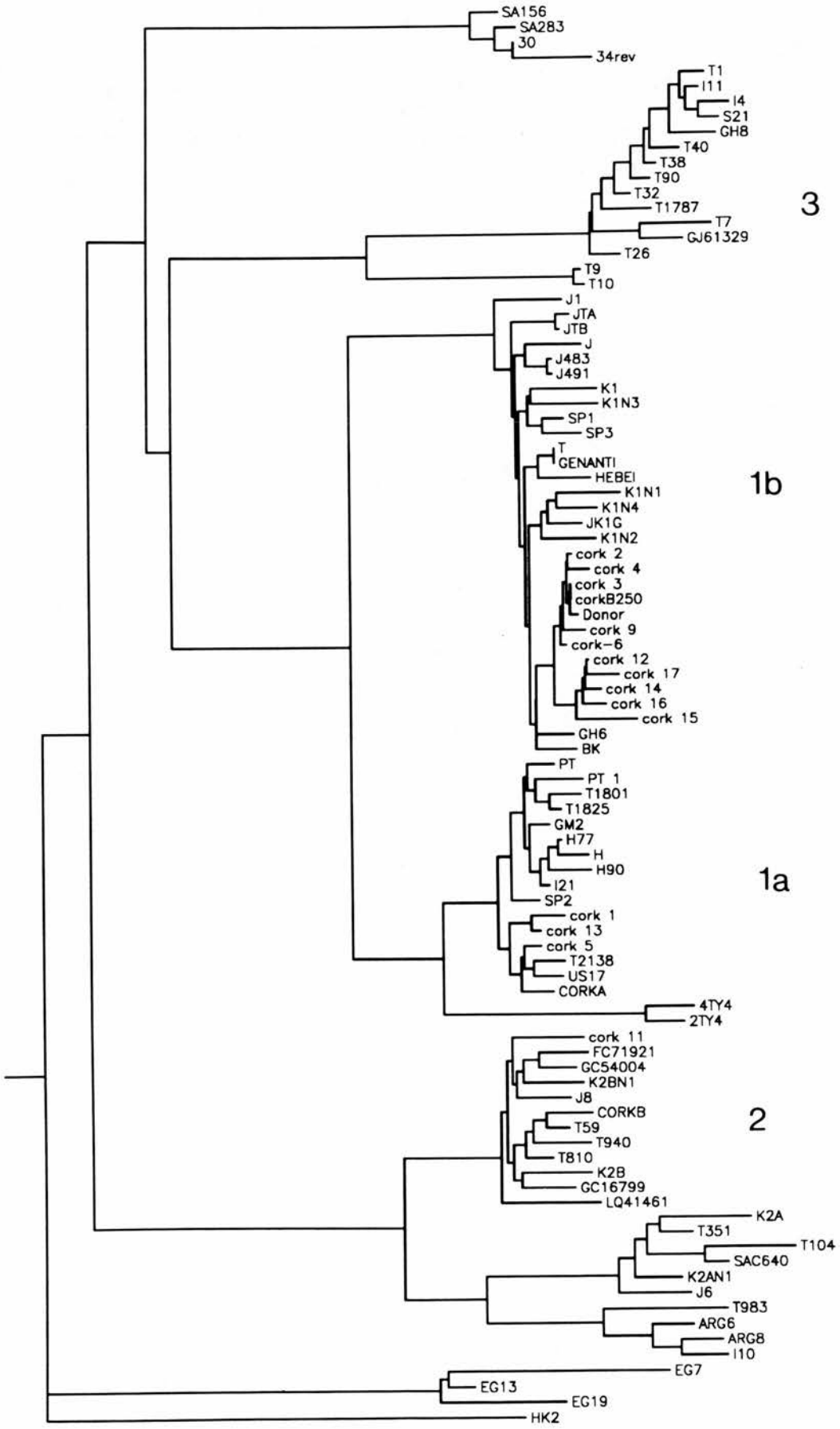
Comparison of nucleotide sequences in the NS-5 region from recipients of anti-D immunoglobulin and blood donors in Eire (all samples prefixed " cork "). Cork 2, 3, 4, 6, 9, 12, 14, 15, 16, and 17 : anti-D recipients. Cork 1, 5, 11, 13, A and B : non anti-D recipient blood donors (A and B donated blood in Dublin, all other samples sequenced were donated in Cork). Cork DONOR : suspected source of HCV infected plasma incorporated in plasma pool used to make batch 250; Cork B250 : sequences from the batch of anti-D IVIG implicated in HCV transmission. " . " indicates identity with J (Kato et al., 1990), nucleotide substitutions are indicated. PT and K2BN1 (Enomoto et al., 1990) are shown for comparison. Nucleotide numbering as in Choo et al., 1991.

Figure 5.2. LEGEND

Phylogenetic analysis of NS-5 sequences, shown as a rooted tree. Nucleotide sequences from recipients of anti-D IVIG group closely together with published sequences of type 1b. Non anti-D recipient blood donor nucleotide sequences group with published sequences of type 1a and 2b. Samples numbered as in fig. 5.1.

Figure 5.2.

PHYLOGENETIC TREE OF NS-5 SEQUENCES



5.3.4. DISCUSSION

Of 897 plasma samples that showed reactivity with antigens in the RIBA-3, 371/577 (64%) were RIBA-3 positive and RT-PCR positive, compared to a figure of 72% in the blood donating population in Scotland (chapter 4, section 4.2.3.(3)). The lower rate of RT-PCR positivity in RIBA-3 confirmed samples in this group may be due to individuals who have cleared the virus but have a persistent antibody response. In a retrospective study of women in East Germany who were infected with HCV by the administration of contaminated anti-D IVIG (Dittmann et al., 1991), anti-HCV was demonstrated in 90% of serum samples collected 6-12 months after infection and 10 years later, 69.2% were anti-HCV positive. It will not be possible to carry out retrospective testing on women infected in Eire since there are no archived plasma samples.

It has been estimated that 8000 women in Eire received anti-D IVIG in 1977, and projecting from current findings approximately 10% of these will now be positive for anti-HCV. The prevalence of anti-HCV among women who received anti-D IVIG in years other than 1977 is much lower at approximately 1%.

The rate of anti-HCV in anti-D recipients 17 years after infection is lower than the 69.2% anti-HCV positive individuals at 10 years after infection reported in East Germany. One explanation for this is that the infecting dose of HCV was higher in East Germany since plasma from 2 HCV infected donors was incorporated into a pool of ten donors which was then used to make different immunoglobulin batches.

In Eire one HCV infected donor contributed to a pool containing 200 donations and used to make a single batch. Fewer vials from the contaminated batch in Eire may have contained HCV (a limiting dilution effect) resulting in fewer women being infected from the one batch. The number of batches containing plasma from the HCV infected donor is currently being investigated, of the four batches available, all were positive for HCV RNA. A common factor between the two transmission events is the manufacturing method used in the production of the anti-D IVIG. Cohn fractionation was not used in either country and the only IgG purification step was passage of the plasma through a DEAE-Sephadex column.

Phylogenetic analysis of NS-5 HCV sequences derived from virus present in the recipients of implicated batches of anti-D IVIG revealed that all sequences grouped very closely together indicating a common origin. Sequences from the HCV infected anti-D donor and the implicated batch of anti-D IVIG also grouped amongst those of the recipients although they were most closely related to each other. The similarity between these sequences was retained despite the 20 year period of separate evolution. Rates of sequence change for HCV have been estimated by studies of HCV infection in humans and experimentally infected chimpanzees (Ogata et al., 1991; Abe et al., 1992; Okamoto et al., 1992c). Different regions of the genome appear to evolve at different rates and variants of the same genotype have been predicted to differ by up to 9% over the complete genome in a 30 year period (pers comm Dr.P.Simmonds, Dept Medical Microbiology, Edinburgh). A study of the evolution of the HCV genome in a chronically infected patient showed a 2.7%

difference between isolates over a 13 year period in the same part of the NS-5 region amplified in this study (Ogata et al., 1991). Pairwise comparison of the donor sequence and recipients, showed differences of 2.7%, 1.8% and 0.4% for recipients cork 15, 17 and B250 respectively, with 0 to 0.9% differences in the other 8 recipient samples. The NS-5 region is thought to code for the virus replicase and is relatively well conserved amongst all viral RNA-dependent RNA polymerases (chapter 3, section 3.3.). It is likely that sequences in this region are conserved to keep the functional domains of these proteins intact.

The genotype of the virus infecting all the anti-D recipients is 1b, a genotype commonly found in Scotland, Europe and also detected in recipients of anti-D IVIG in Germany (Höhne et al., in press). Sequences obtained in the same region of the HCV genome from 6 blood donors infected via other parenteral routes grouped separately with previously published sequences for genotypes 1a and 2b. This confirms that the anti-D recipients are infected with virus that is phylogenetically distant from other HCV isolates in Eire.

There have been few reports of the persistence of HCV viraemia over long periods of time. The majority of studies correlating viraemia and antibody response have been on blood donors where the route and time of exposure is often unknown (Bresters et al., 1993; Tobler et al., 1994) or on patients with symptomatic chronic hepatitis due to HCV infection via blood transfusion (Wang et al.,1992; Lelie et al., 1992). Further studies on these women who were infected 20 years ago will provide

valuable information on the epidemiology and natural course of HCV infection since the date and time of exposure is known and it should be possible to make estimates of the approximate infective dose of HCV received. All women who are found to be RT-PCR positive are to be counselled and offered liver biopsy with the intention of offering interferon treatment to those showing histological evidence of chronic hepatitis.

The results obtained in the studies reported here (sections 5.2. and 5.3.) and the East German report indicate that HCV can be transmitted by IVIG preparations, in a manner similar to HCV transmission by coagulation factor concentrates. A recent report by Yei et al. (1992) described how cold ethanol fractionation of a plasma pool, prepared exclusively form anti-HCV antibody reactive donations resulted in detectable levels of HCV RNA in Fraction II, the fraction used for IVIG preparation. In general, licensed IVIG preparations have a good record for safety, but the report by Yei et al. and the work presented here indicates that routine anti-HCV screening of plasma donors and the incorporation of specific procedures that are virucidal in IVIG manufacture are essential to prevent any further cases of transmission of NANBH by IVIG preparations. There is always the possibility that a failure may occur in The Good Manufacturing Practice (GMP) but even then HCV screening of plasma donors would increase the safety of the product by reducing the possibility of contamination of fractionated products by HCV infected plasma.

CHAPTER 6

6. PARVOVIRUS

6.1. GENERAL INTRODUCTION

Parvoviruses are DNA viruses that infect a number of animal hosts and humans may become infected with parvovirus B19. Infection in normal indivduals causes mild flu-like symptoms but in those with underlying haemolytic disease it can lead to aplastic crisis as the virus targets erythroid precursor cells (Morfini et al., 1992). Transmission of parvovirus B19 by blood and blood products has been reported (Mortimer, 1983). Standard immunological screening methods are not useful for screening blood donations for B19; detection of virus nucleic acid is an alternative, however at present there is no suitable technique for screening large numbers of blood donations on a routine basis. In the following sections, the physical and epidemiological characteristics of B19 are considered which highlight the problems associated with prevention by iatrogenic transmission. A method for screening a large number of samples for the presence of B19 virus nucleic acid is described and applied to the screening of blood donations.

6.2. THE VIRION

The parvovirus family consist of a large number of physically and chemically similar viruses which infect many animal species (table 6.1.). The parvovirus virion has a relatively simple structure and is among the smallest of the DNA animal viruses. The particle has icosahedral symmetry, a diameter of 18 to 26 nm and is non-enveloped. When purified from human plasma the B19 virion is found to contain two major capsid proteins of 83 and 58 kilodaltons, of which the latter is the predominant

Table 6.1.

PARVOVIRUSES OF VERTEBRATES

Autonomous Subgroup	
Rat Virus	RV
H-1 Virus (rat)	H-1
RT Virus (rat)	RT
TVX (unknown)	TVX
Minute Virus of Mice	MVM
LuIII (unknown)	LuIII
B19 (human)	B19
Porcine Parvovirus	PPV
Bovine Parvovirus	BPV
Feline Parvovirus	FPV
Species host range variants:	
Mink Enteritis Virus	MEV
Canine Parvovirus	CPV
Racoon Parvovirus	RPV
Lapine Parvovirus	LPV
Aleutian Disease Virus (mink)	ADV
Goose Parvovirus	GPV
Possible members:	
Minute Virus of Canines	MVC
HB Virus (human?)	HB
RA-1 (human)	RA-1
Dependovirus Subgroup	
Adeno-associated type 1 (monkey)	AAV-1
Adeno-associated type 2 (human)	AAV-2
Adeno-associated type 3 (human)	AAV-3
Adeno-associated type 4 (monkey)	AAV-4
Adeno-associated type 5 (human)	AAV-5
Bovine Adeno-associated Virus	BAAV
Canine Adeno-associated Virus	CAAV
Avian Adeno-associated Virus	AAAV
Possible members:	
Equine Adeno-associated Virus	EAAV
Ovine Adeno-associated Virus	OAAV

species. Approximately 80% of the mass is protein and the remainder is DNA. The buoyant density of the intact virion in caesium chloride is 1.39 - 1.42 g/cm^3 which is due to the relatively high DNA to protein ratio. The particles do not appear to contain lipids, carbohydrates, cellular or virally coded enzymes, or low molecular weight histone-type proteins. The virion is extremely resistant to inactivation, being resistant to extraction with lipid solvents, pH ranges between 3 and 9, heating to 56^{0}C for 60 minutes and exposure to relatively high salt concentrations (which are used in caesium chloride isopycnic gradients). The stability of the particle is probably due to its relatively simple structure. However, the virus can be inactivated by formalin, β-propiolactone, hydroxylamine and oxidising agents; prolonged storage and repeated freeze-thawing may compromise virus integrity resulting in reduced stability to extremes of temperature or salt concentration although remaining infectious.

6.3. THE GENOME

6.3.1. DNA STRUCTURE AND SEQUENCE

The B19 genome appears to have features in common with both autonomous and helper dependant viruses, sequencing studies have shown the genome to be highly conserved (Mori et al., 1987). Computer analysis of the putative viral polypeptides from MVM(p), AAV2 and B19Au have identified extensive regions of homology between the polypeptides encoded by all three genomes. However B19 replicates in erythropoietin-stimulated human bone marrow cultures without the aid of a helper virus which suggests it is probably an autonomous virus. For the purposes of this

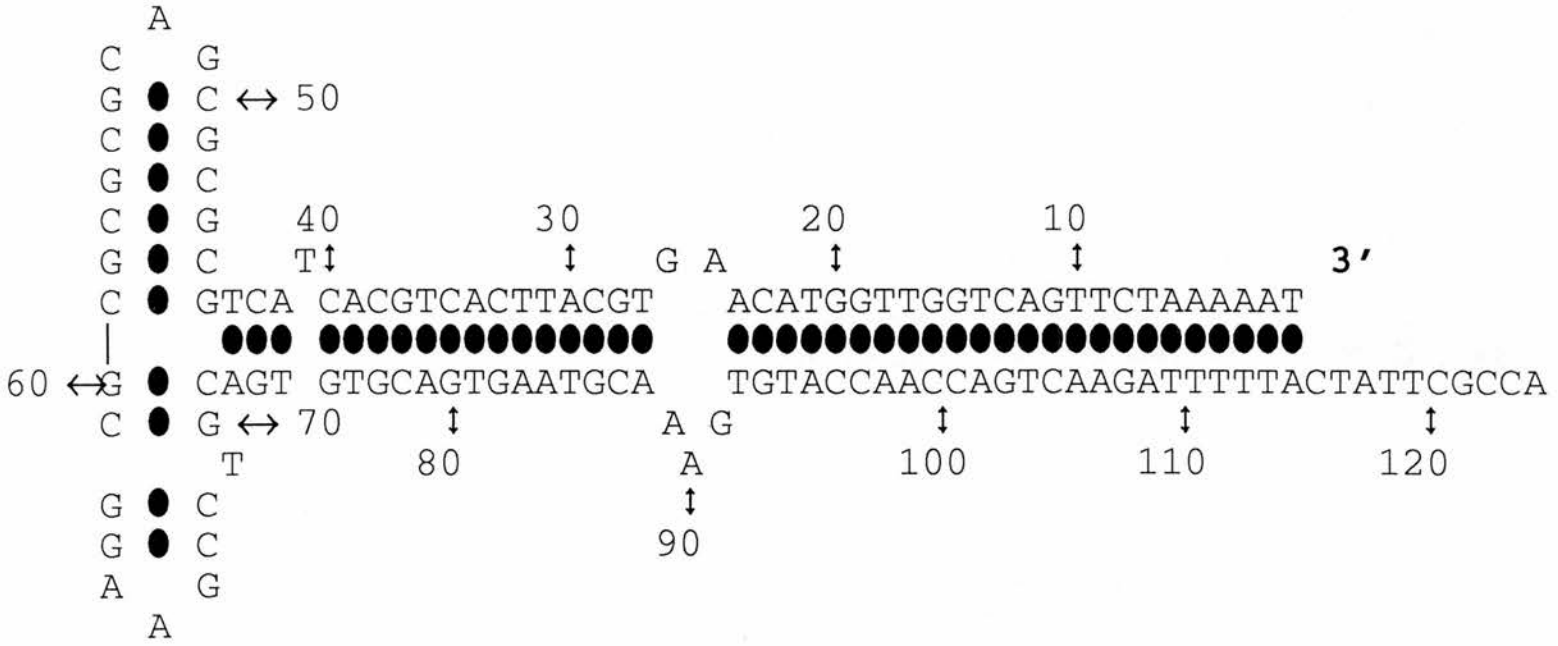
chapter B19 is considered to be an autonomous virus. The autonomous parvovirus virion contains a single-stranded polydeoxynucleotide chain of M.W. 1.55 - 1.97 x 10^6 daltons (Crawford et al., 1969; Gardiner et al., 1988). Several parvovirus DNAs have been sequenced and in general the DNA molecule is about 5 kilobases in length, H-1 and MVM being 5176 and 5084 nucleotides respectively, (Astell et al., 1983; Astell et al., 1986; Sahli et al., 1985; Rhode et al., 1983), AAV2 DNA contains 4,680 bases. Strands of one polarity, those that are complementary to the mRNA, are encapsidated in the autonomous parvoviruses (Bourguignon et al.,1976) while strands of both polarities are encapsidated with equal frequencies in AAV strains (Mayor et al., 1969). Since the study presented in this chapter is on parvovirus B19 the human autonomous parvovirus infection, discussion of virus structure and replication will be directed towards autonomous virus particles. Adeno associated virus should be considered separately as sufficient differences exist for it to remain outwith the scope of this chapter.

Both the 3′ and 5′ termini of all parvovirus genomes analysed to date contain palindromic nucleotide sequences which can exist in the form of relatively stable hairpin duplexes in the single-stranded form of viral DNA. At the 3′ end of the virion strand of most autonomous parvovirus DNAs the palindromic sequence is approximately 115 bases long (Salzman et al., 1970). In the human parvovirus B19, these sequences appear to be more than 300 nucleotides in length. This segment of the DNA can fold back on itself to form a hairpin structure stabilised by hydrogen bonding between the self-complementary sequences fig.6.1. Comparison of the 3′

nucleotide sequence of MVM(p), H-1, KRV and H-3 have been made (Astell et al., 1979), although there are some minor differences, the sequence of the first 150 nucleotides of each virus is essentially the same. Since sequence homology at this level is not maintained throughout the viral DNA, this suggests a strong pressure to conserve this particular region. A total of 104 of the first 115 nucleotides of MVM can be base-paired to form a stable Y-shaped hairpin, it is possible that is the constraint implied in maintaining this overall conformation which resists genetic drift, rather than absolute linear sequence requirements. A bubble in the duplex stem created by a mismatch between nucleotides 23 to 26 and 88 to 91 is a common feature in all four viruses which suggests that it may also be an essential element of the terminal structure (fig 6.1.). Of particular note is the fact that for each DNA sequenced almost every base pair in the short arms of the T structure is a G•C base pair. The 5′ end of the human B19 genome appears to be unique in that its nucleotide sequence is a terminal repeat of the sequence at the 3′ end, where as in other autonomous parvoviruses the 5′-terminal sequence is also palindromic and can form a hairpin, but the sequence is completely unrelated to that at the 3′ end. The 5′ terminal sequence is not a perfect palindrome (as found at the 3′ end), there are short, self-contained internal palindromic sequences. Two sequences that represent an inversion of the terminal segment of the genome are found with equal frequency, these arise from the fact that the 5′-terminal hairpin is not perfectly symmetric.

Terminal sequence organisation gives valuable information on the correct mechanism of DNA replication and thus mature virion formation.

Fig 6.1. Hairpin loop structure formed by the 3′ nucleotides of B19 DNA.



6.3.2. ORGANISATION OF THE GENOME

In all parvoviruses analysed to date, all of the protein coding regions appear to be clustered on one of the DNA strands, which is, by definition, the plus strand. The autonomous parvovirus genome contains two large ORFs. The first spans map position (mp) 6 - 42 (at the left half of the genome) and encodes two non-structural (NS) proteins NS1 and NS2. Mutations occurring within this ORF block viral replication and gene expression. The ORF spanning mp 45 - 90 (covering much of the right half of the genome) encodes the coat proteins. Autonomous parvoviruses contain three coat proteins (the exception being Aleutian disease virus ADV which contain only two coat proteins). VP1 (M.W. 80000 - 86000) and VP2 (64000 - 75000) have identical amino acid sequence except for additional amino acids at the NH2 terminus of VP1. This implies that synthesis of VP1 involves splicing to add a second (smaller) ORF to the 5' end of the major ORF. VP3 is the major coat protein (except in ADV) (M.W. 60000 - 62000) and represents approximately 80% of the total mass.

6.4. PRODUCTIVE INFECTION

6.4.1. THE PRODUCTIVE LIFE CYCLE

The autonomous parvoviruses display a high degree of host specificity and replication is tightly regulated by the parvovirus itself. B19 virus shows a strict tropism for erythroid precursor cells, and it has been established that the target cells of B19 are limited to the erythroid lineage, the susceptibility of which increases with differentiation (Takahashi et al., 1990). The productive life cycle is always lytic, and

DNA replication takes place in the nucleus. For parvovirus replication to occur it is necessary for infected cells to be in S phase of division as parvoviruses do not have the ability to stimulate or turn on host DNA synthesis (Walter et al.,1980).

The productive infection is initiated by absorption of the virion to specific cell-surface receptors. It is thought that the receptors involve or are displayed on a protein backbone. The receptor for a particular virus sero-type could be a single molecular species of a number of different molecules which carry specific carbohydrate chains. There is little information regarding the next stages of infection. Translocation of the virion across the plasma membrane occurs and cytoplasmic virus becomes clustered within multivesicular bodies and heterophagosomes arranging themselves in an array lining the membrane in aggregates. The capsid is thought to play a role in the initiation of viral gene expression so it is likely that the virion arrives in the nucleus as an intact entity.

It is yet to be determined whether the parvovirus B19 genome becomes integrated into the host cell DNA or not. The MVM genome does not integrate into the host DNA however it is not known what occurs during prolonged nonlytic interactions such as restrictive infections. In contrast AAV genomes integrate with high efficiency. The fact that B19 have closely related terminal repeat sequences at each end of their genomes, rather than having unique termini as in MVM and H-1, suggests that integration may be possible.

6.4.2. AUTONOMOUS VIRUS DNA REPLICATION

Viral DNA replication occurs in three distinct phases :

(i) synthesis of a parenteral complementary DNA strand

(ii) amplification of duplex DNA forms

(iii) excision and concomitant packaging of progeny single-stranded viral DNA.

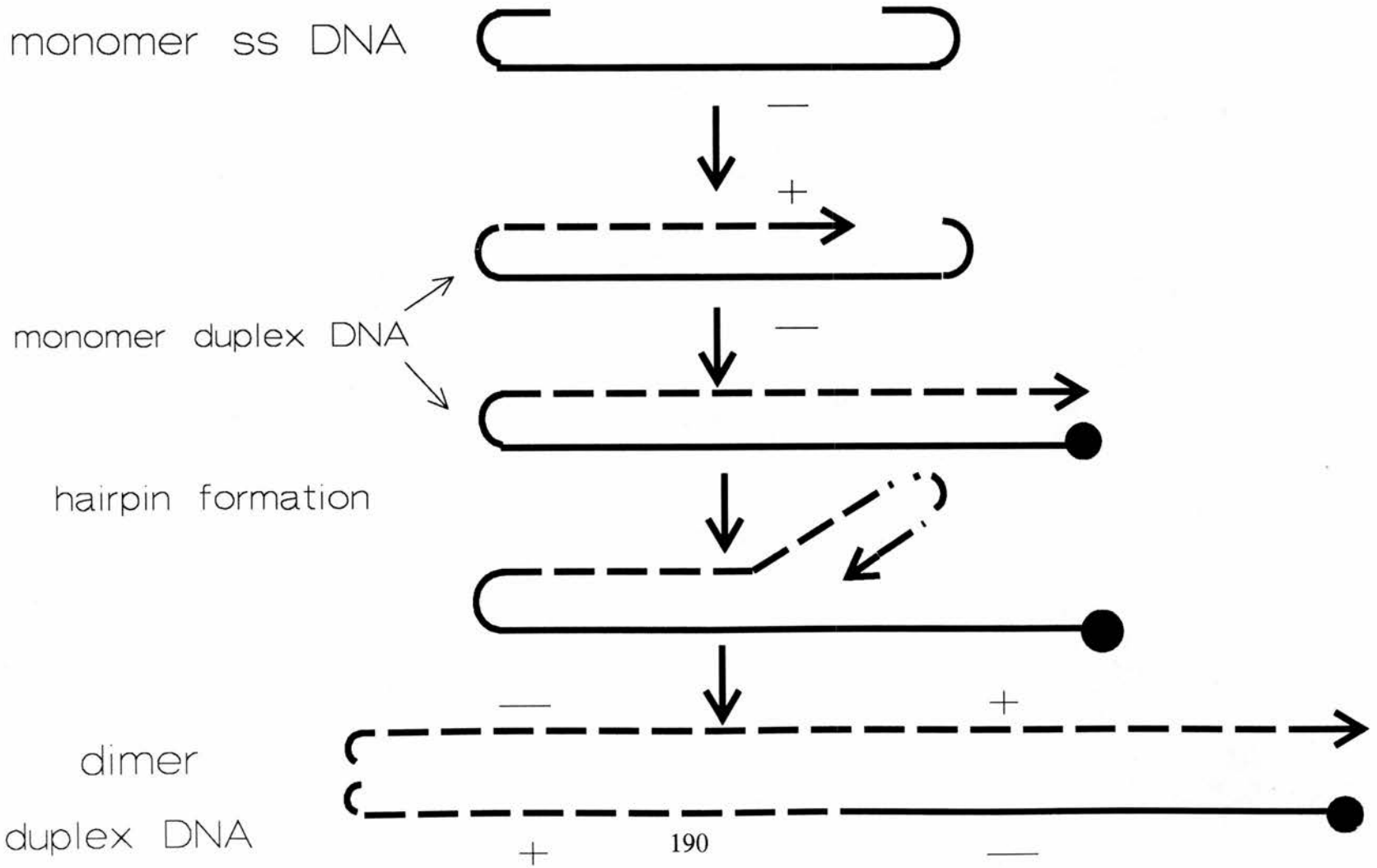
Viral and cellular replication are closely associated, cellular DNA polymerases are used, although it is yet to be defined whether the involvement of cellular DNA polymerase α or δ or a combination of both (and the participation of β) are active in replication. For polymerisation to take place a primer with a 3′OH group in addition to a template is necessary for enzyme action. As mentioned in section 6.2.1. the terminal sequence of the autonomous parvovirus genome are palindromic. In order for the linear DNA genome to replicate the 3′ terminus of the virion strand folds back on itself and thus serves as a primer, with the complement of the 5′ terminus (ie: the 3′ terminus of the complementary strand) forms a similar hairpin structure providing a primer.

The replication events have been proposed by Astell et al. modified by Tattersall and Cotmore, as a model for autonomous parvovirus replication. However the events for human B19 replication are probably more complex due to the repetition of terminal nucleotide sequences. The determination of the details of replication would require

the development of an in vitro system for DNA replication which is hampered by the presence of the palindromic 3′ terminal hairpin primer. This structure could potentially be used as a primer by almost any DNA polymerase in vitro and thus the signal-to-noise ratio would be very small.

A simplified version of the overall scheme is presented in fig. 6.2.. The scheme outlined is typical of viruses such as MVM and H-1 which package predominantly (>99%) single sense DNA strand which is the complement of that encoded into RNA, and are thus (-) strand DNA viruses. However studies have shown that LuIII and B19 encapsidate both strands with approximately equal efficiency (Cotmore and Tattersall 1984; Bates et al., 1984). Selection of strands for packaging appears to be a virus-coded rather than a host-specified function. However, few of the preformed viral capsids are used to package DNA and mature into infectious virus particles (as demonstrated in *in vitro* virus host cell systems), a mixture of empty capsids and full virions is released from the cell following nuclear degeneration and rupture of the plasma membrane.

Figure 6.2. DNA FOLDING IN PARVOVIRUS REPLICATION



6.5. HUMAN PARVOVIRUS INFECTION

Infection with the human parvovirus B19 is common with approximately 50% of adults having antibody. It is known to cause several pathological conditions and can become a persistent infection (Frickhofen and Young, 1989). Infection of healthy individuals is generally asymptomatic, B19 can cause non-specific respiratory tract infection and is usually spread by the respiratory route. Acute infection in children results in "fifth disease" or erythema infectosium (Anderson et al., 1984), in adults it manifests as an arthralgia-arthritis syndrome (White et al., 1985). In patients with underlying haemolytic disease, transient aplastic crisis or anaemia may result (Pattison et al., 1981; Serjeant et al., 1981). The aplastic crisis results from a halt in erythropoiesis in the bone marrow for five to seven days. Individuals with a normal immune system clear the virus, however infection of the immunocompromised can cause pure red cell aplasia and bone marrow failure (Kurtzman et al.,1989; Kurtzman et al. 1987). The underlying cause is the weak humoral immune response in such patients. Administration of intravenous immunoglobulin generally assists in transient or permanent clearance of the virus and improvement of the anaemia. Studies have shown that in utero infection of the foetus in the second or third trimester may result in hydrops fetalis (Anand et al., 1987; Porter et al., 1988). The hydrops may be transient and is often associated with anaemia and sometimes with myocarditis in the foetus, or ultimately fatal. It has not yet been established whether B19 infection is a cause of infrequent anatomical or functional abnormalities such as congenital red cell aplasia or central nervous sytem disorders.

Although B19 is spread via the respiratory route, parenteral transmission is possible via transfusion of blood products made from plasma pools containing blood donated during the viraemic stage of B19 infection. Recent evidence shows that parvovirus B19 can be transmitted by coagulation factors VIII and IX (Mortimer, 1983; Williams et al., 1990; Bartolomei Corsi et al., 1988; Lyon et al., 1989; Azzi et al., 1992). Haemophiliacs receiving coagulation factors prepared from large plasma pools show a higher prevalence of anti-B19 antibodies than individuals of the same age. In addition, some haemophiliacs are HIV infected, which may result in them being immunocompromised and therefore at increased risk if receiving a contaminated batch of factor concentrate.

6.6. DETECTION OF PARVOVIRUS B19

B19 was first discovered in 1974 by Cossart et al. while carrying out routine evaluation on tests for hepatitis B surface antigen (HBsAg). Current biophysical methods for detection of B19 vary in specificity, sensitivity and ease of application. To date B19 has been identified by electron microscopy (E.M.) (Bond et al., 1986; Clewley et al., 1987) radioimmunoassay (RIA) (Westmorland and Cohen 1991), counterimmunoelectrophoresis (CIE) (Cohen et al.,1981), nucleic acid hybridisation (Nascimento et al.,1991; Anderson et al., 1988; Porter et al., 1988; Salimans et al., 1989a,b) and polymerase chain reaction (PCR) (Clewley, 1989; Salimans et al., 1989a,b). In the clinical situation routine diagnosis is made on the detection of IgM only, however in immunocompromised patients serological assays may not be

informative as the serum concentration of B19 antigen and specific antibodies may be too weak to be detectable.

At present routine serological diagnosis of B19 infection is hampered by the availability of viral antigen. Recent attempts to produce sufficient antigen have included transfection of B19 DNA into Chinese Hamster Ovary cells (CHO) or Cos-7 cells (Kajigaya et al., 1989), production of recombinant antigen in E.coli (Sisk and Bergman 1987) and in baculovirus (Kajigaya et al.,1991). Tissue culture systems using infected early gestation aborted foetal liver for primary culture production (Brown 1991) provide higher levels of B19 DNA released into the supernatant than previously reported, greater than 1μg/ml compared with 20 ng/ml (Yaegashi et al., 1989). Bone marrow culture systems provide one hundred fold less (Ozawa et al., 1987). It is unlikely that a tissue culture system will provide a source of diagnostic antigen as foetal tissue is not readily available, cultures are viable for a short time and the technique is labour intensive. Recently Satoshi et al. (1992) reported propagation of B19 in an erythropoietin-dependent strain of megakaryoblastic leukemia cell line called UT-7. Virus was detected in cultures for up to three months at approximately 1000 copies per cell which at optimal multiplicity of infection was 20 to 50 fold greater than that amount of virus inoculated. This particular cell line may prove to be more useful in the production of infectious virus and in the analysis of B19 persistence, cytotoxicity and permissivity.

Practically it is more probable that the use of synthetic B19 peptides will provide larger amounts of antigen for diagnostic assays. In particular, enzyme-linked immunoabsorbent assay (ELISA) for detection of B19 specific IgM and IgG (Fridell et al., 1989; Schwarz et al., 1991a). Currently the development of peptides for immunogenetic reaction are those of the major structural proteins VP1 (Kajigaya et al., 1991) and VP2 (Fridell et al., 1989). A recently developed assay making use of a synthetic VP2 peptide showed the degree of specificity and sensitivity of the peptide antigen to be in the region of 90% (Fridell et al., 1991). Considering the evidence that it appears to be appropriate to test for IgM in diagnosis of recent B19 virus infection then the IgM peptide assay would be a choice for diagnosis of B19 infection in acute-phase serum samples.

However IgM detection is really only useful in determination of recent viral infection by which time the patient is usually symptomatic. In addition, not all patients can produce IgM or IgG, for instance those with congenital or acquired immunodeficiency and patients with chronic haemolytic anaemia become symptomatic during early viral infection prior to antibody production, the immature immune system of the foetus only starts to produce antibody at eighteen weeks gestation and then only in small amounts. Even if tests based on other sources of antigen were introduced, native B19 antigen would still be required to provide reference assays. Detection of the presence of B19 DNA and hence viraemia remains the definitive diagnosis of infection. Dot-blot hybridisation techniques are generally difficult to perform, require the use of radioisotope and are less sensitive than the

PCR. Although PCR cannot distinguish between degraded or intact infectious viral DNA, it may be of considerable benefit in screening plasma donations prior to pooling for factor concentrate production.

6.7. BLOOD DONOR SCREENING

6.7.1. INTRODUCTION

There is a seasonal pattern to B19 infections. In temperate climates it is endemic throughout the year with infection occurring most frequently in late winter, spring, and early summer months. Long-term cycles of B19 infection have been observed in the U.K. to occur at 4 to 5 year intervals (information supplied by the Centre for Disease Control, Scotland.).

Previous attempts at screening blood donors for B19 infection have concentrated on identifying a donation that could be used as a source of viral antigen for diagnostic serology and secondly to establish the incidence of viraemia in the donating population (Cohen et al., 1990). In order to prevent any viraemic donations being used it is necessary to use rapid screening techniques that can be applied to a large number of samples on a daily basis. Cohen et al. (1990) screened 24000 donor samples from January to May 1990 using a dot-blot hybridisation assay and counter-immunoelectrophoresis (CIE). The dot-blot hybridisation assay was found to be too labour intensive and time consuming and was not used further after screening 6500 donations.

The PCR has previously been applied for detection of B19 in clinical samples (Clewley, 1989; Salimans et al., 1989a,b; Koch and Adler, 1990; Cassinotti et al., 1993). A study was undertaken to measure the frequency of B19 infection in the donating population, to determine the feasibility of using the PCR to routinely exclude B19 from blood products and to identify a highly viraemic donation that would provide a source of antigen.

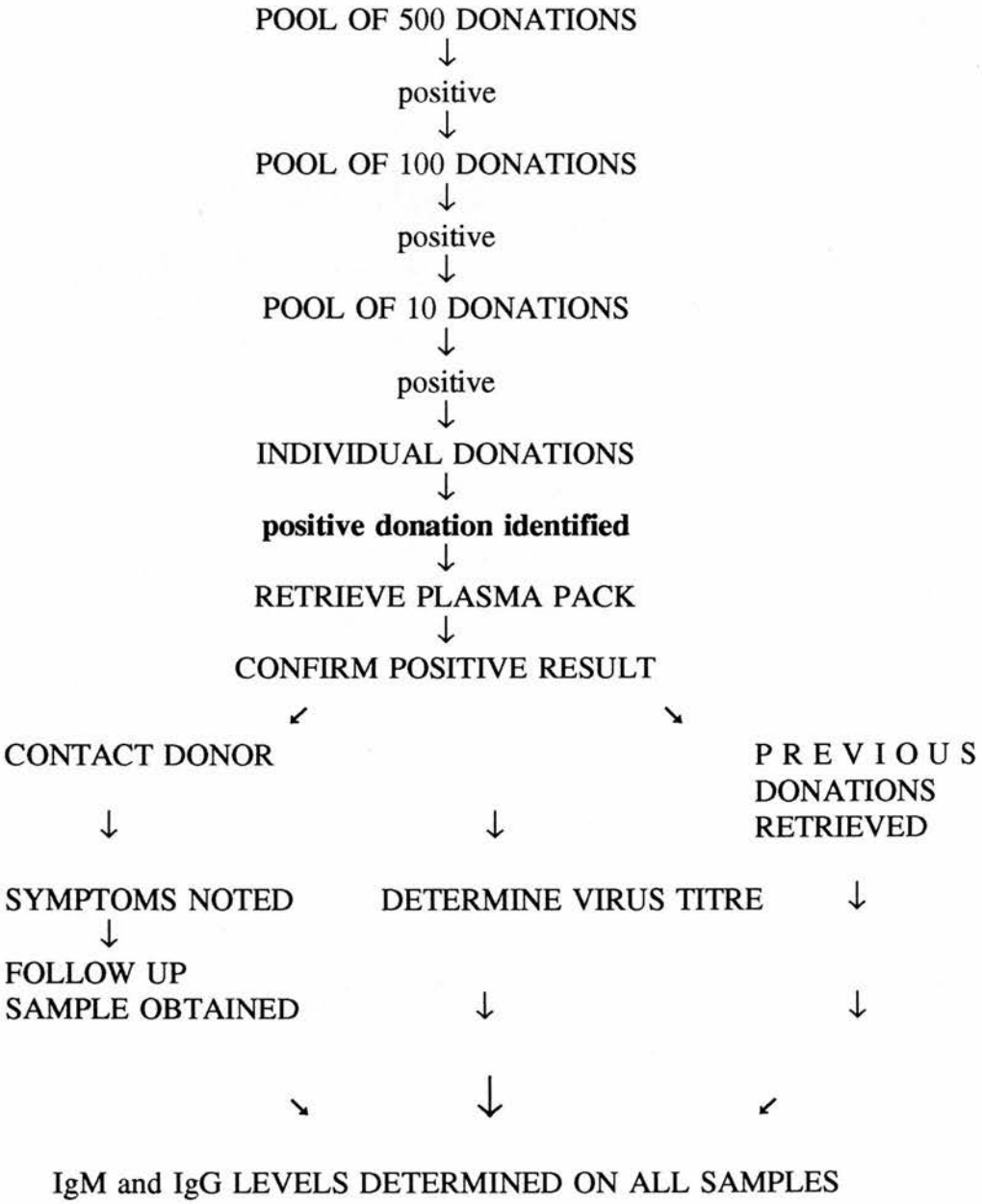
6.7.2. SAMPLES

Blood from 20000 volunteer donors in the Edinburgh area was collected over a three month period from May to July 1991. 200µl aliquots of each donation was collected in a microtitre plate, with pools of 100 donations being made at the time of aliquoting and stored frozen at -20^0 C, these aliquots were then recombined in pools of 500 donations and centrifuged followed by DNA extraction as described in chapter 2, section 2.2.. Samples making up the pools of 500 donations that were positive by PCR, were reconstituted successively in pools of 100, 10 and 1 single donation in order to identify the viraemic donor. Following identification of a positive blood donation the plasma pack was retrieved and an aliquot tested by PCR, any previous or follow-up donations were identified and tested by PCR. B19 quantification was carried out by titration at limiting dilution, with 5 to 15 replicates tested at each 10 fold dilution (chapter 2, section 2.9., Simmonds et al., 1990a). On confirmation of a positive PCR result the donor was contacted and B19 positive donors were asked to attend an interview, at which time they were questioned about any symptoms they

may have experienced and a blood sample was requested at this time. Fig.6.3. is a flow chart showing the course of action.

The screening process was repeated in 1993. The main objective at this time was to screen all donations that would be incorporated into a plasma pool used to produce a batch of Factor VIII concentrate that would be B19 free. Donations were screened at two different periods in the year in order to compare the incidence of viraemia. Six thousand donations were screened in March 1993 and a further 6000 donations in August and September 1993. During both collection periods in 1993 plasma packs were segregated and stored at -40^{0}C at the Protein Fractionation Centre prior to Factor VIII production. Ten thousand donations are usually sufficient to produce a batch of Factor VIII, however it was necessary to screen 12000 donations as not all donations are used for concentrate production, in addition some packs burst accidentally.

Figure 6.3.


POOL OF 500 DONATIONS
positive
POOL OF 100 DONATIONS
positive
POOL OF 10 DONATIONS
positive
INDIVIDUAL DONATIONS
positive donation identified
RETRIEVE PLASMA PACK
CONFIRM POSITIVE RESULT
CONTACT DONOR
PREVIOUS DONATIONS RETRIEVED
SYMPTOMS NOTED
DETERMINE VIRUS TITRE
FOLLOW UP SAMPLE OBTAINED
IgM and IgG LEVELS DETERMINED ON ALL SAMPLES

6.7.3. PRIMERS

Primer sequences are shown in chapter 2, section 2.6., table 2.2.. Primers PV1 to PV4 were derived from the previously published sequences of Wi and Au variants of human parvovirus B19 (Blundell et al., 1987; Shade et al., 1986). This region showed substantial nucleotide and sequence homology with adeno-associated virus (Srivastava et al., 1983), minute virus of mice (Astell et al. 1983), H1 (Rhode and Paradiso, 1983) and feline parvovirus (Carlson et al., 1985). Primer sequences derived independently for this study were similar in position to those used by Clewely (Clewley, 1989) and represent one of the most highly conserved regions of different parvovirus genomes. Primers PV1 and PV2 were used in the first round of a nested PCR followed by amplification with PV3 and PV4 in the second round stage. Sufficient volume of this second round product was made to allow for direct sequencing to be carried out. Primers PV5 to PV8 amplified part of the genome encoding an antigenic region within the B19 nucleocapsid (Fridell et al. 1989), PV5 and PV6 were used in the first round reaction then PV7 and PV8 used in the second reaction. DNA extraction, PCR method and sequencing reactions are described in chapter 2, sections 2.2, 2.6, and 2.8. respectively.

6.7.4. SEROLOGY

Plasma samples from PCR positive blood donations, stored previous donations and follow up samples from infected donors obtained at recall were tested blind using capture radioimmunoassay (Cohen et al., 1983). The tests were carried out by Dr.

Bernard Cohen and staff at The Virus Reference Laboratory, Central Public Health Laboratory, Colindale, London.

6.8. RESULTS

6.8.1. BLOOD DONOR SCREENING

Screening of 20,000 donations from volunteer donors in the Edinburgh and South East Scotland area in May to July 1991 resulted in the identification of 6 B19 positive donations. In March 1993 and September 1993 screening of 6,000 donations at each time resulted in the identification of 23 and 0 B19 positive donations respectively. The limit of sensitivity of the screening method was 5 virions per ml in a pool, and therefore 2,500 virions per ml in each component unit. All six donations were positive with both primer sets, PV1 to PV4 and PV5 to PV8. The procedure used in identifying a positive donation is shown in fig. 6.3. and 6.4. A positive PCR result obtained in a pool of 500 donations was traced to an individual donation by sub-dividing the initial pool into 5 pools of 100, a positive in this round of screening indicates the 10 pools of 10 donations to be investigated, and each set of 10 donations produced a single PCR positive sample thus identifying the viraemic donor. The six packs of plasma corresponding to the positive samples in 1991 were recalled from the Protein Fractionation Centre in Edinburgh, and all were confirmed positive by PCR. A similar approach was taken in 1993, all screened packs were segregated at the Protein Fractionation Centre, testing of the recalled packs confirmed a positive donation in all 23 donations. The remaining B19 free packs are to be used to manufacture a B19 free batch of factor VIII concentrate.

Figure 6.4.

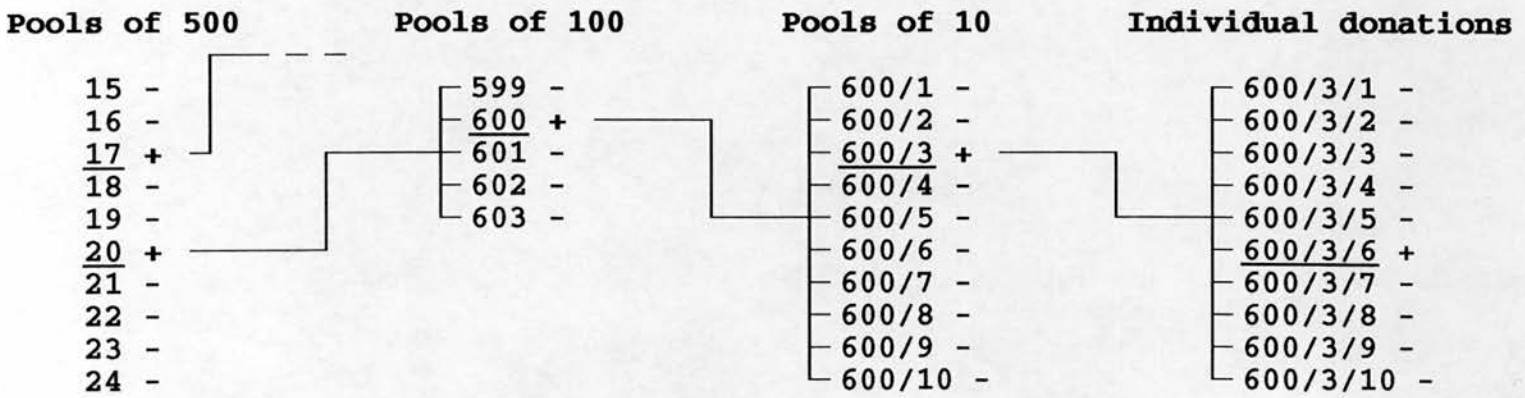


Figure 6.4. LEGEND : Example of the identification of B19-infected donors by sequential PCR screening. Positive pools of 500 donations (nos.17 and 20 underlined) were tested in five pools of 100 units. Identification of pool 600 allowed rescreening in pools of 10 (showing that 600/3 was positive) and finally the identification of the infectious donor (600/3).

6.8.2. SEQUENCING

The nucleotide sequences of the six B19 positive donations identified in 1991 were determined using DNA amplified with primers PV1 and PV2, and also PV5 and PV6. The nucleotide sequence obtained from the six donors differed from published sequences of B19 and from each other by 1 to 6 substitutions with one exception. Two donors yielded the same viral sequence, on recall these two donors were found to be husband and wife. The fact that different viral sequences were detected in each donor confirms that the positive result obtained on screening is a true positive result and not inadvertent contamination of samples or buffers. The nucleotide sequences obtained are shown in table 6.2., and are compared with the B19-Au variant (Shade et al., 1986).

6.8.3. CLINICAL AND VIROLOGICAL RESULTS

Previously donated blood samples and those obtained as follow-up samples as well as the actual donation were tested for B19-specific IgG and IgM and for B19 DNA by PCR (fig 6.5.). Five of the six donors, p1 to p5, were negative for both IgM and IgG in all samples prior to donation, and four of the five were still negative at the time when viraemia was detected. When recalled these four donors were found to be IgG positive and IgM positive 3 to 6 months after donation. Samples from p6 were IgG positive before, during and after donation, with no B19-specific IgM detected in follow-up samples from this particular donor. Unfortunately p5 did not wish to attend for follow-up interview consequently there is no post donation sample available. B19 DNA was undetectable in all samples collected from donors on recall

Table 6.2. VARIATION IN NUCLEOTIDE SEQUENCE OF B19 DNA AMPLIFIED FROM VIRAEMIC BLOOD DONORS

Nucleotide Position	B19-AU	Nucleotide change					
		p1	p2	p3	p4	p5	p6
Region 1							
1503	A		G(-)	G(-)			
1530	A		G(-)	G(-)	G(-)	G(-)	G(-)
1576	G(V)	T(L)					
Region 2							
3172	G				A(-)	A(-)	
3182	T(S)	C(L)	C(L)	C(L)	C(L)	C(L)	C(L)
3214	T		A(-)	A(-)			
3223	T	C(-)	C(-)	C(-)	C(-)	C(-)	C(-)
3307	G	A(-)				A(-)	
3355	C	T(-)	T(-)	T(-)	T(-)	T(-)	T(-)
3394	A	G(-)					G(-)

Variation in nucleotide sequence of B19 DNA amplified from viraemic donors. Region 1 between nucleotides 1415 and 1584, numbered as in Shade et al., 1986, amplified by primers PV1 and PV2. Region 2 between nucleotides 3118 and 3444, amplified by primers PV5 and PV6. Standard single letter codes used in parentheses to indicate variable amino acids: V:valine, L:leucine, S:serine (-):indicates a silent nucleotide substitution.

which was generally 60 to 100 days after donation, in addition blood donated previously by all six donors was PCR negative.

Symptoms experienced varied greatly between each individual. The two female donors (p1 and p2) reported rash, fever and arthropathy. The male donors were generally asymptomatic, including p5, who had extremely high levels of circulating virus (table 6.3.). Two of the donors were husband and wife (p2 and p3), and they reported parvovirus-like symptoms of infection in their children.

Figure 6.5. TIME COURSE OF THE APPEARANCE OF ANTI-B19 SPECIFIC IgM AND IgG

Graph indicating the time course of the appearance of anti-B19-specific IgM and IgG in donors identified as viraemic on screening by PCR. Symbols: O anti-B19 IgG and IgM negative; ● anti-B19 IgM and IgG positive; ● anti-B19 IgG positive and IgM negative. All donors were PCR negative for B19 DNA in samples before and after donation.

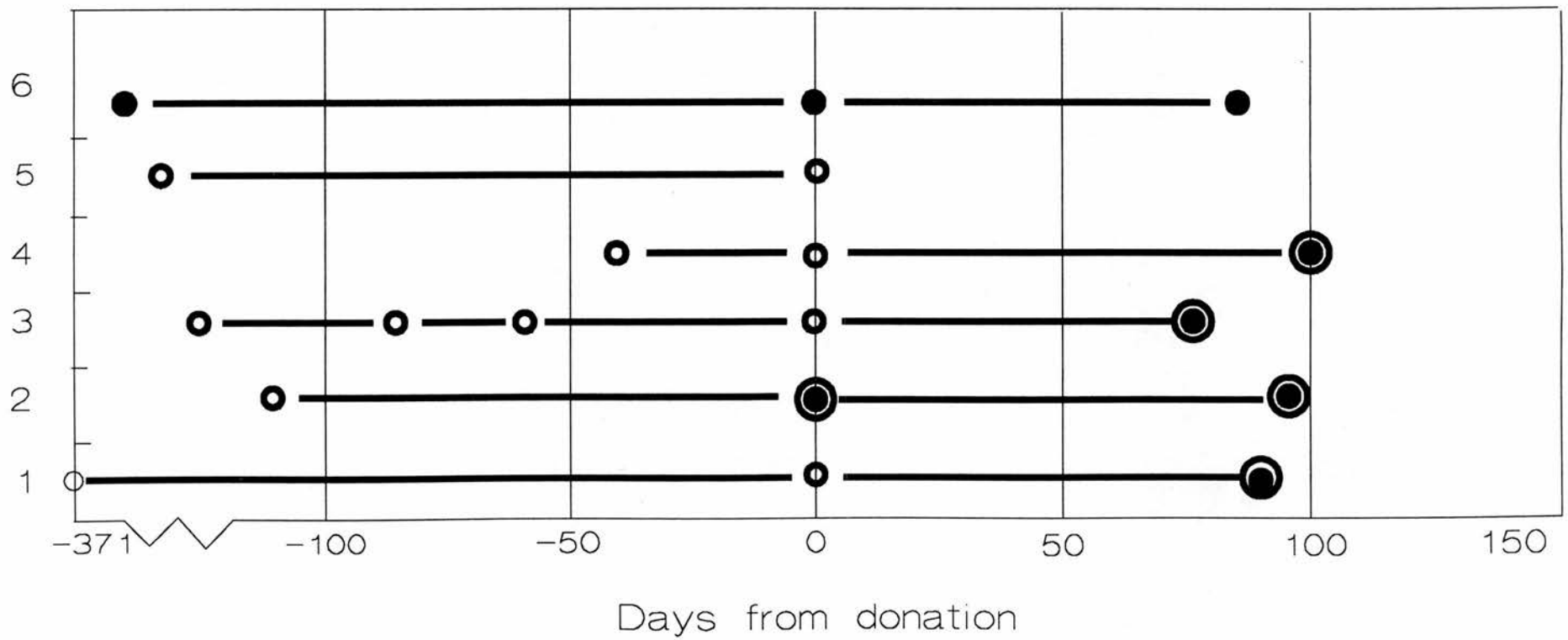


Table 6.3.

CLINICAL SYMPTOMS AND VIRAL TITRES IN VIRAEMIC BLOOD DONORS

Donor	Age	Sex	Symptoms	Copies of B19 DNA /ml
1	19	F	Rash, fever, arthropathy 14 days post-donation	3.8×10^5
2	35	F	Rash, fever, arthropathy 5-6 days post donation Rash recurred after one month	2.6×10^4
3	36	M	Asymptomatic	3.8×10^4
4	30	M	Asymptomatic	2.0×10^7
5	36	M	Asymptomatic	2.0×10^{11}
6	35	M	Tired with occasional joint pains several months before and after donation	5.0×10^4

6.9. DETECTION OF PARVOVIRUS B19 IN BLOOD PRODUCTS

6.9.1. INTRODUCTION

Pools of 3,000 to 10,000 plasma donations are used as the raw material in the manufacturing process for many blood products (chapter 1, Introduction). The frequency of PCR-positive donations detected in the studies carried out in 1991 and 1993 of 0.03% and 0.4% respectively indicates that B19 might frequently contaminate such blood products. Infection of the immunocompromised or a haemophiliac with B19 through administration of these blood products may have severe consequences (section 6.5.). B19 has no lipid envelope and is stable to most chemical and physical treatments widely used in inactivation processes to eliminate lipid enveloped viruses. There remains the possibility that B19 may persist in the final product. Several blood products were tested for the presence of B19 DNA and the effect of heat treatment on its survival in Factor VIII preparations was investigated.

6.9.2. SAMPLES

Blood products were obtained from the U.K. National Institute of Biological Standards and Controls (NIBSC) and the Protein Fractionation Centre (PFC), Scottish National Blood Transfusion Service (SNBTS), Edinburgh. Preparation and storage of Factor Concentrates is described in chapter 2, section 2.1. and 2.2.. Eighteen non-heat-treated batches of factor VIII, 9 non-heat-treated batches of factor IX and 10 batches of IVIG were tested by PCR using primers PV1 to PV4. To investigate the kinetics of B19 inactivation during heat treatment, intermediate-purity

factor VIII was spiked at a ratio of 1:100 with B19-positive plasma, freeze dried, and heated using model SNBTS production cycles. Additional levels of such proteins in intermediate-purity factor VIII have been shown previously to have no effect on the freeze-drying or heating characteristics of this product (pers comm Dr. Helena Hart, PFC, Edinburgh).

6.9.3. RESULTS

Of the factor VIII and IX, 13 and 7 respectively were found to be PCR positive, while none of the batches of IVIG contained detectable amounts of B19 DNA (table 6.4a.). Five of the PCR-positive batches of factor VIII were heat-treated by a standard manufacturing process of 80^0C for 72 hours and retested. Two of the five batches remained PCR positive. The B19 DNA titre was determined before and after heat treatment on the five available batches. In the two batches that remained PCR positive there was at least 90% reduction in the amount of detectable viral DNA (table 6.4b.).

To investigate the kinetics of virus inactivation, a batch of factor VIII was spiked at a ratio of 1:100 with plasma from donor p5. During the lyophilisation step, the viral DNA titre declined from 4.5×10^8 to 7×10^7 copies per ml. The lyophilised material was then subjected to dry-heat-treatment for 24, 48 and 72 hours at 80^0C. A reduction to a final titre of 8×10^6 copies per ml was achieved with this process (table 6.4c.). The overall reduction in the viral DNA titre is comparable to that observed on heat treatment of nonspiked samples (table 6.4b.).

Table 6.4a.
FREQUENCY OF DETECTION OF PARVOVIRUS B19 IN BLOOD PRODUCTS

Blood Product	Source	H.T.	No. pos.	No. tested
FACTOR VIII:	NIBSC	No	4	8
	SNBTS	No	7	10
	SNBTS	Yes	2	5
FACTOR IX:	NIBSC	No	3	4
	SNBTS	No	4	5
Intravenous IgG	SNBTS		0	10

Table 6.4b.
EFFECT OF DRY HEAT TREATMENT AT 80°C FOR 72 HOURS ON LEVELS OF PARVOVIRUS B19 DNA IN FACTOR VIII

Batch	B19 DNA, copies/ml		%
	before	after	Reduction
1	500	< 22	>95 %
2	50	< 22	>55 %
3	340	< 22	>93 %
4	1900	170	91 %
5	1400	50	96 %

Table 6.4c.
KINETICS OF REMOVAL OF B19 DNA DURING HEAT TREATMENT OF FACTOR VIII

Sample	Virus titre copies/ml	% Reduction
Spiked factor VIII	2.5×10^8	
After lyophilisation	10^8	60%
24 hours 80°C (dry heat)	2.5×10^7	90%
48 hours	2.5×10^7	90%
72 hours	8×10^6	97%

6.10. DISCUSSION

6.10.1. DETECTION OF PARVOVIRUS B19 INFECTION IN BLOOD DONORS

The incidence of parvovirus B19 viraemia in healthy blood donors in the studies undertaken in both 1991 and 1993 (0.03% and 0.4% respectively), was considerably higher than that reported by previous surveys in which antigen detection methods were used (Cohen et al., 1990; Mortimer, 1983). One possible explanation for this increased rate is the increased sensitivity of the PCR which is capable of detecting 5 copies per ml of plasma. Hybridisation methods typically detect 0.1 to 1pg of B19 DNA which is equivalent to approximately 2 X 10^4 to 2 X 10^5 copies per ml. An improved hybridisation assay with a chemiluminescent probe showed a sensitivity of 20fg of B19 DNA, or 4,000 copies (Musiani et al., 1991). While this method would have detected all six donations in this study (unlike the former) it would still not have been suitable for testing large pools due to its lack of sensitivity compared to the PCR. Using the PCR it was possible to carry out direct sequence analysis of the amplified DNA and to make a positive identification of B19 sequences and distinguish B19 from other parvoviruses (or contaminants). Minor variations in sequence between B19 in different blood donations confirmed that five independent B19 infections in six donors had been identified. Sequence variability in the VP2 region was comparable to that observed among other epidemiologically unrelated isolates of B19 (Umene and Nunoue, 1991).

All the donors identified as being viraemic in the 1991 study were asymptomatic at the time of donation, and three of the four male donors remained asymptomatic. However both female donors reported symptoms of rash and arthropathy in the following 5 to 14 days post-donation. A higher rate of symptomatic infection in women has been reported previously (Woolf et al., 1989). Four of the five donors recalled showed a specific IgM and IgG response to B19 following donation which is consistent with primary infection (Anderson et al., 1985; Cohen et al.,1983). One donor p6 was unusual in that he was anti-B19 IgG positive before donation and before the time of viraemia and no IgM was detected at or after the time of donation. One possible explanation would be that this individual was reinfected with parvovirus; it has previously been shown that experimental inoculation of a healthy adult volunteer with a low level of anti-B19 IgG led to reinfection, manifested by a second IgM response and increased levels of anti-B19 IgG (Anderson et al., 1985). However, neither serological response was observed in donor p6 in this study. Of interest is that this individual had complained of recurrent polyarthralgia and flu-like symptoms for several months both before and after donation. It is possible that he had a chronic, low-grade infection with subclinical reactivation of B19 at the time of donation.

6.10.2. PARVOVIRUS CONTAMINATION OF BLOOD PRODUCTS

Parvovirus B19 DNA was detected in two-thirds of the batches of noninactivated clotting-factor concentrates tested. Considering the frequency of viraemic donors, the size of the pools used for manufacture and the fractionation process used (Schwarz

et al., 1991b) then this level of contamination could be expected. Although parvovirus B19 DNA can be detected by PCR this does not necessarily imply infectivity and more precise studies of B19 inactivation by different treatment regimes will need to be carried out in the future using recently developed in vitro culture systems (Brown et al., 1991; Schwarz et al., 1992). Whether these blood products are infectious will depend on the stability of the virus, the possible neutralisation of infectivity by mixture with antibody from other units in the pool (Kurtzman et al., 1989), and the partitioning of the virus during cryoprecipitation and cold ethanol precipitation during manufacture. However, these results contrast with studies on other viruses, such as hepatitis C virus, in which the same treatment eliminated infectivity and completely destroyed all traces of viral nucleic acid (Garson et al., 1990a).

Several reports have provided serological evidence for transmission of B19 by non-heat-treated factor VIII and prothrombin complex concentrates (Mortimer, 1983; Rollag et al., 1991; Williams et al., 1990). Clinical data on recipients of " virally inactivated " concentrates is contradictory and possibly reflects the variation in the precise conditions used for virus removal. In one study, serological testing of haemophiliacs who had received dry-heat-treated (72 hours at 80^0C) factor VIII showed that they had no increased risk of infection over age-matched controls (Williams et al., 1990). However, elevated rates of infection were found in recipients of dry heat-treated or steam-treated factor VIII (Bartolomei Corsi et al., 1988). In addition, previously nontransfused haemophiliacs were reported to become acutely

infected 7 to 14 days after the first infusion of detergent-treated (Azzi et al., 1992; Morfini et al., 1992) or heat-treated (Azzi et al., 1992; Bartolomei Corsi et al., 1988; Lyon et al., 1989) clotting factor.

6.10.3. DONOR SCREENING BY PCR : FEASIBILITY OF ROUTINE TESTING FOR PARVOVIRUS B19

In order to identify a PCR-positive blood donation it was necessary to carry out four sequential separate DNA extraction and nested PCR amplification reactions. In the 1991 study, in the initial pool of 500 donations the plasma that was found to be negative (34 out of 40 tested) could have been safely transfused after only one amplification reaction. An insignificant proportion of units (54 out of 20,000) would need to subjected to four screens by PCR to rule out contamination. In this particular study, no attempt was made to optimise the time taken for DNA extraction and PCR. Despite this, it was possible to retrieve all six units of infected plasma from the PFC before manufacture. Similarly in the 1993 study it was possible to retrieve all 23 positive packs identified and parvovirus DNA was confirmed in all. However it proved to be more problematic to retrieve all previous and follow-up donations for this particular study.

In theory, because the DNA extraction and amplification steps could be carried out in a single working day, it should also be possible to prevent transfusion of all contaminated units of erythrocytes and platelets without causing a significant delay to the time of issue. Routine application of this method for virus detection in donor

centres should be possible in the future with the development of rapid DNA extraction methods and automation of amplification reactions which are currently being developed.

CHAPTER 7

7. THE ROLE OF PCR IN THE DETECTION OF VIRUSES IN BLOOD AND BLOOD PRODUCTS

7.1. GENERAL INTRODUCTION

At present the diagnosis of microbial infection is generally based on the detection of a specific antibody or antigen response. However detection of nucleic acid is becoming widely used with the advent of recombinant DNA technology which has enabled the production of nucleic acid probes for many microorganisms, including, viruses, bacteria, fungi, spirochetes and rickettsia (Tenover, 1988). Culture methods for diagnosis are often difficult or require long periods of cell growth to obtain results. In addition, there may been a long window period (3 to 12 months) between viraemia and antibody response, which is a specific problem in the detection of HIV or HCV. Two methods of nucleic acid detection widely used are PCR and/or hybridization. The use of nucleic acid hybridization for microbial diagnosis has been limited by its insensitivity compared to immunological tests. However with the development of the PCR, microbial nucleic acids may be " enriched " prior to detection and a new level of sensitivity be obtained. Quantification of the amount of the replicating virus is possible once the nucleic acid has been isolated. The ability to detect small amounts of virus makes the PCR test prone to false-positive results due to contamination of the specimen with the DNA to be detected and has made the technique difficult to automate. False-negative results are rare, and are mainly due to the presence of inhibitors of DNA polymerase in the specimen. Incorporation of appropriate positive and negative controls allows for monitoring of these false reactions. In addition, it has proved difficult to standardise the extraction and RT-

PCR reactions (Zaaijer et al., 1993). Commercial kits are now available that enable the detection by PCR of HCV, HIV and HBV virus particles in plasma and serum samples.

A limitation to the number of samples that can be tested by PCR is the need to extract nucleic acid from each sample to be tested. In the research situation this may not represent a significant problem but for the routine screening of blood donors this and the difficulty in automating the PCR has prevented the technique from becoming widely used for the screening of blood donations. One way of overcoming the problem associated with extraction is the pooling of samples to enable the screening of a large number of samples. Such a method was developed and used to screen blood donations for parvovirus B19 (McOmish et al., 1993b and chapter 6). The following sections describe the extension of the pooling technique to other viruses thus offering a step towards PCR screening of a large number of samples on a routine basis. Comparison of a commercial HCV PCR detection system is made with the RT-PCR technique described and used in this work (chapter 2, sections 2.5 and 2.6). The commercial kit and the RT-PCR technique are compared and validated using a reference panel from the Eurohep Group whose aim is to standardise the PCR used in laboratories across the world.

Manufacturers of blood products are required to prove the safety of their preparations using validated fractionation and virus inactivation techniques. Infectivity assays are cell culture based systems that are widely used for the detection of replicating virus

particles and are often employed in studying virus inactivation procedures. However, it takes several weeks to obtain a result from a cell culture system, whereas a PCR result may be available within 2 days and can be performed on a larger number of samples. The role of PCR as a replacement for infectivity assays in studying virus inactivation is considered.

7.2. SCREENING OF BLOOD AND BLOOD PRODUCTS FOR HEPATITIS A VIRUS

7.2.1. INTRODUCTION

Transmission of hepatitis A virus (HAV) to haemophiliacs by factor VIII concentrates purified by ion-exchange chromatography and virally inactivated by solvent-detergent (SD) treatment has been reported (Mannucci et al., 1992; Gerritzen et al., 1992; Temperely et al., 1992; Peerlink et al., 1993). Transmission may have occurred because HAV is a non-lipid enveloped virus and would not be destroyed by the SD treatment. In addition, blood donation are not routinely screened for HAV antibody. Normann et al. (1992) reported that HAV RNA could be detected in two of four batches of factor VIII (produced by the S/D process) tested by PCR and one of the four PCR-positive batches had been administered to four haemophiliacs who had acute HAV infection following factor VIII treatment. Since many manufacturers use the SD method of virus inactivation a study was undertaken to examine the presence of HAV RNA in factor VIII concentrate produced by the SD method Chromatography material used in the purification steps was also examined since it

is possible that HAV could preferentially adsorb to the chromatography material and be subsequently eluted.

Using the pooling method described in chapter 6, 12,000 blood donors were tested for HAV infection by RT-PCR to measure the prevalence of HAV positive individuals in the donating population in Scotland.

7.2.2. SAMPLES

Batches of high-purity factor VIII concentrate manufactured by the Scottish National Blood Transfusion Service (SNBTS) and the New York Blood Centre were studied (table 7.1.). In these preparations the SD process was incorporated into the manufacturing procedure. In addition, three batches of high-purity factor VIII, one manufactured by Octapharma, and implicated in HAV transmission (Peerlink and Vermylen, 1993), and two manufactured by Biotransfusion, but not implicated in the HAV transmission, were studied. These batches were also prepared by the SD method. Three batches of high-purity factor VIII manufactured by the Bioproducts Laboratory (Elstree, UK) using a high-purity monoclonal antibody purification method after SD treatment were also tested. All factor VIII preparations were supplied as lyophilised powder and reconstituted prior to testing according to the manufacturer's instructions with final concentrations of factor VIII ranging from 25 to 100IU/ml.

Samples of chromatography gel used in the purification of factor VIII, the gel material and a 2M sodium chloride (NaCl) eluate from it were obtained from Biotransfusion, Lille (courtesy of Dr. T. Burnouf). Other samples were chromatography gel that had been used in the purification of ten production batches of factor VIII by the SNBTS, and a 2M NaCl and 50mM NaOH eluate.

Twelve thousand volunteer blood donations collected in Scotland at two time points in the year (March and August 1992) were tested in pools of 100 donations as described in chapter 6, section 6.7.2..

Twenty five coded batches of factor VIII concentrate (Melate) were also provided by the New York Blood Centre. These batches had been produced using a mixture of plasma from local volunteer donors and cryoprecipitate obtained from a commercial company.

Table 7.1. BATCHES OF HIGH-PURITY FACTOR VIII CONCENTRATE STUDIED

Preparation	Manufacturer	Number of Batches	HAV RNA PCR Result	Production Method
H8	SNBTS (UK)	28	negative	SD
Melate	New York Blood Center (USA)	9	negative	SD
Factor VIIIconc.SD	Octapharma (Austria)	1	negative	SD
Factor VIIIconc.SD	Biotransfusion (France)	2	negative	SD
8SM	Bioproducts (UK)	3	negative	monoclonal antibody purification & SD
Melate	New York Blood Centre (USA)	25	3/25 positive	(mixed cryoppt. in some batches)*

* Cryoprecipitate is the cold-insoluble portion of plasma remaining after fresh frozen plasma has been thawed between 1 and 6°C.

7.2.3. PCR AND PRIMERS

Reverse transcription and PCR were carried out as described in chapter 2, sections 2.5 and 2.6.. HAV specific oligonucleotide primers (chapter 2, section 2.6., table 2.4.) were designed from a consensus sequence of 6 HAV sequences in the 5′ NCR which is the most conserved region of the genome in all isolates. 5′ nucleotide positions are based on the wild type HM-175 strain (Cohen et al., 1987). Validation of the HAV RT-PCR was based upon a panel of 26 plasma samples from patients with suspected hepatitis A infection (kindly provided by Dr.S.Burns, City Hospital, Edinburgh). Five out of 7 samples that were IgM anti-HAV positive were positive by RT-PCR; all the remaining samples (7 IgM anti-HAV weak positive; 7 Ig M anti-HAV negative; 5 IgG anti-HAV positive) were all HAV negative by RT-PCR. HAV RNA could also be detected in 1 sample of liver tissue from a patient with fulminant hepatitis A who required a liver transplant. Direct nucleotide sequence analysis was carried out on PCR products from three of the RT-PCR positive plasma samples and deduced sequences were 100% identical to published HAV sequences. These plasma samples were used as positive controls in further experiments.

The sensitivity of the PCR assay for the detection of HAV RNA in factor VIII concentrates was determined by spiking experiments. HAV RNA copy number was determined in a serum sample of a patient with hepatitis A infection by titration at limiting dilution (chapter 2, section 2.9.). Five to fifteen replicates were tested at and around the cut-off point of a serial 10-fold dilution set, and the copy number calculated as described in chapter 2, section 2.9.. Varying volumes of this serum

were added to a factor VIII concentrate reconstituted according to the manufacturers instructions to 25IU/ml, previously tested and shown to be negative for HAV. The number of copies of HAV RNA was determined by titration at limiting dilution and compared to that obtained with the original serum sample used for spiking.

7.2.4. RESULTS

HAV RNA could not be detected by PCR in any of the batches of factor VIII preparations studied (table 7.1), including the one batch of factor VIII that was implicated in hepatitis A transmission in Belgium (Peerlink and Vermylen, 1993). Also, HAV RNA could not be detected in the chromatography gel before or after use in the purification of factor VIII, or in the eluates from these gels. A total of 12,000 blood donor plasma samples were tested as pools for HAV. No positive donations were detected at either period of collection.

The failure to detect HAV RNA in any of the batches screened had two implications: either it was not possible to detect HAV in factor concentrates in this way or the batches tested were free from contamination with HAV. To continue the search for HAV in factor concentrate, several batches were obtained from a manufacturer outwith Europe. Two sets of batches of Melate were tested blind (table 7.1.). Two out of 15 vials proved to be positive for HAV RNA, these were identified as batches 91C and 91D. A further 10 vials were tested and 2 positive vials were identified as batches 91B and 91C. A positive hybridisation signal was obtained on a filter carrying PCR product from each of the three positive batches when incubated with

a HAV-DIG labelled probe (fig 7.1; method: chapter 2, section 2.10.). In addition, direct sequence analysis of PCR product from the three positive batches was carried out. Sequences obtained showed 99% homology to the wild type HAV HM-175, the only differences being an A→T change at position 177, and a T→A change at position 204 (numbering from wild type HAV HM-175, Cohen et al., 1987). Both of the batch sets tested included all three PCR positive batches but batches 91B and 91D were not PCR positive in set one and set two respectively, whereas batch 91C contained 100% cryoprecipitate obtained from a commercial source and was PCR positive in both sets tested. Batches B and D were composed of 14% commercial cryoprecipitate and 86% volunteer donor derived material.

A detection limit of HAV RNA was calculated by spiking experiments with human plasma to be 266 copies of HAV RNA per ml of factor VIII concentrate. As an independent test, factor VIII concentrates were spiked with a cell-culture-adapted strain of HAV (HM175/18f) that had been titred by an infectivity assay on African green monkey kidney cells by Dr. Stanley Lemon (University of North Carolina) and obtained courtesy of Dr. Henrietta Margolis-Nunno (New York Blood Centre). A factor VIII sample containing 50 radioimmunofocus assay units (RFU)/ml (Lemon et al., 1991) was PCR negative, whereas samples containing 5,000 and 500,000 RFU/ml were PCR positive consistent with the estimated detection limit.

Figure 7.1.

HYBRIDISATION SIGNAL OBTAINED WITH HAV PCR PRODUCT

1. Positive control (IgM anti-HAV positive, RT-PCR positive plasma sample)
2. Negative control (HCV RT-PCR positive plasma sample)
3. Batch 91C - from first test set
4. Batch 91D - from first test set
5. Batch 91B - from second test set
6. Batch 91C - from second test set
7. Melate batch 91C - uncoded vial
8. Positive control (as no.1)

7.2.5. DISCUSSION

Reports of transmission of HAV by blood products have been rare and limited to single donor products used in blood transfusion (Barbara, 1982; Skidmore et al, 1984). In 1992 and early 1993 there were four reports of HAV infection in haemophiliacs treated with factor VIII concentrates produced from plasma using ion-exchange chromatography with SD treatment as the virus inactivation method (Mannucci, 1992; Gerritzen et al., 1992; Temperely et al., 1992; Peerlink et al., 1993). Although in these cases the starting plasma was from a variety of different sources the manufacture was performed by or under licence from a single company. These HAV transmission reports caused some concern about the efficacy of the SD treatment method in the inactivation of HAV in the manufacture of factor VIII concentrates. In Scotland, factor concentrates produced using the SD method of virus inactivation have been used since 1991.

The study was undertaken to establish if HAV RNA was present in factor VIII concentrates produced using SD as the method of virus inactivation and to assess the risk of HAV infection in Scottish haemophiliacs who are receiving this product. HAV RNA could not be detected in any of the factor VIII concentrates or chromatography gel eluates studied, suggesting that the presence of HAV RNA in factor VIII preparations is a rare event, consistent with the sporadic nature of the reported transmission episodes. The negative results are also consistent with the lack of detectable cases of HAV viraemia in the 12,000 blood donations tested.

An alternative explanation for the negative RT-PCR results could be that the detection limit for HAV RNA was not sufficiently low to detect an infecting HAV dose. The detection limit established for the RT-PCR used in this study was 266 copies of HAV RNA per millilitre of reconstituted factor VIII concentrate solution. The concentration of factor VIII in a single experiment was 25 IU/ml, which is equivalent to a detection limit of approximately 10 copies of HAV RNA per IU of factor VIII. Another explanation might be that HAV RNA was at limiting dilution, ie: present in some vials in batches of factor VIII concentrates, and not in others. Each batch of factor VIII concentrate is manufactured from plasma derived from 10,000 or more donations and if one donation from a viraemic donor was present in the pool with a hypothetical HAV titre of 10^6 copies/ml, there would be a titre of 10^2 copies/ml or less in the final plasma pool which would be below the sensitivity of the RT-PCR used in this study. This uncertainty would be best resolved by quantitative studies of the level of HAV RNA present in batches of factor VIII concentrate that were definitely implicated in transmission.

The detection of three HAV RNA positive batches of Melate demonstrates that it is possible to identify the virus in batches of factor concentrate. The fact that batch 91C was PCR positive in both test sets while batches 91B and 91D were only found to be positive once is consistent with virus being present at limiting dilution in some batches.

The failure to detect HAV RNA in any of the production batches of factor VIII concentrate and none of the pools from 12,000 blood donors suggests that factor VIII concentrate produced using the SD method of virus inactivation is unlikely to transmit HAV. This is confirmed by retrospective testing of stored samples showing that there have been no seroconversions amongst Scottish haemophiliacs since the introduction of SD treated products (pers comm Dr. H. Watson, Royal Infirmary, Edinburgh). Therefore the reported cases of HAV transmission by factor VIII concentrates may actually represent community acquired infections. The susceptibility of haemophiliacs to HAV infection may have increased also, due to falling levels of passive immunity previously provided by anti-HAV IgG present in intermediate-purity factor concentrates (ie: concentrates prepared prior to 1991 in which dry heat treatment was the viral inactivation method used which did not remove all protein components) or because of falling antibody levels in the community in general. Reports from around the world indicate that the age distribution of antibody to HAV has changed over the last 10-20 years (pers comm Dr. Gillon, SNBTS, Edinburgh), in that since 1970 childhood infection has become much less common in most " Westernised " countries, the exact timing of this change being different in different countries, and between different regions within the UK. This change in age distribution has two implications for the transmission of HAV by blood products. First, " herd " immunity is reduced, resulting in a reduction in the amount of protective antibody in the donor pool and possibly an increase in transmission of HAV by factor VIII concentrates. Secondly, the number of viraemic individuals in the donating population will be increased as the age of infection shifts from

childhood to young adulthood. Hepatitis A viraemia can be detected by RT-PCR prior to clinical manifestations of disease and up to seven days after clinical onset and the appearance of IgM-HA antibody (Yotsuyanagi et al., 1993). It is therefore possible that blood donations may be taken in this asymptomatic period while the individual is viraemic. The combination of the three factors of possible failure to inactivate the virus during manufacture and the potential increase in incidence of viraemia and loss of protective antibody in the donating population may be contributing elements which have resulted in the transmission of HAV to haemophiliacs through factor VIII concentrates. In the future it may be necessary to boost antibody levels in the donor population, either by selecting naturally immune donors for plasmapheresis or by boosting non-immune donors with vaccine to overcome this problem.

7.3. VALIDATION OF THE HCV PCR AND COMPARISON WITH A COMMERCIAL HCV PCR KIT

7.3.1. INTRODUCTION

The detection of HCV by RT-PCR is now widely used for the diagnosis and confirmation of HCV infection in the clinical situation. A positive PCR result may prove infection when a serological test gives an ambiguous result or when a patient fails to mount an antibody response. Detection of HCV RNA may sometimes be the only test available for diagnosis, for example, in patients with immune deficient syndromes such as hypogammaglobunaemia. Alternatively a negative PCR result may indicate that viraemia has resolved in a person with detectable HCV antibodies.

Many different HCV RNA extraction methods are used in different laboratories but the most commonly used are guanidine thiocyanate denaturation followed by phenol/chloroform extraction (Chomczynski and Sacchi, 1987) or proteinase K digestion followed by phenol/chloroform extraction (described in chapter 2, section 2.4.). Some laboratories carry out a centrifugation step prior to extraction to concentrate virus. Amplification of the 5'NCR is generally used for detection of HCV, however many different primer sets have been described within this region and it is possible that some genotypes may fail to be amplified by primers that include polymorphic sites between the different genotypes (Wolff et al., 1992). The detection of different HCV genotypes and subtypes and the possibility that their response to interferon treatment may vary (Dusheiko et al., 1994) increases the need to develop a reliable, reproducible and standardised RT-PCR system since PCR results have consequences for patient management and monitoring.

A European group on viral hepatitis (" Eurohep ") aims to standardise HCV RNA detection. A panel of well characterised fresh frozen plasma samples was sent to 30 hepatitis research laboratories in Europe, 7 in the USA and 1 in Japan. Each laboratory detected HCV RNA by its own protocol and the results were sent to the coordinating centre where each laboratory was assigned a code to ensure confidentiality. Results were analysed and reported back to the participants. The correlated results have been published (Zaaijer et al., 1993), the following section reports the results obtained from testing the panel by the RT-PCR method described in chapter 2, section 2.3.; 2.4.; 2.5.; 2.6.. In addition, the samples were tested by a

new commercially available HCV PCR detection system (Amplicor) manufactured by Roche. Comparison of the sensitivity and specificity of this kit with the " in house " RT-PCR system was made. A standardised kit system would be the first step to obtaining a uniform test for HCV RNA on a world wide basis.

7.3.2. SAMPLES

Eurohep samples were shipped frozen and coded to Edinburgh. The test panel consisted of 22 samples and included 10 undiluted blood donor samples with and without HCV infection and two 10-fold dilution series of 6 samples each. The panel was tested using the guanidine thiocyanate and the proteinase K extraction methods, followed by RT-PCR using primers in the 5'NCR (chapter 2, section 2.3.; 2.4.; 2.5.; 2.6.), as well as by the Roche Amplicor test. RT-PCR results from 48 Scottish blood donor samples with known RIBA-3 status were compared with the Roche Amplicor kit results.

7.3.3. THE ROCHE AMPLICOR HCV DETECTION KIT

All tests were carried out according to the manufacturers instructions. Briefly, target RNA was prepared using a guanidine thiocyanate extraction method, HCV target RNA was reverse transcribed and amplified in a single tube reaction using TtH DNA polymerase. Biotinylated primers in the 5'NCR region were used with the incorporation of dUTP in the reaction mix to prevent carryover of contaminants in the PCR reaction. The products of the PCR were denatured and hybridised to a 5' NCR oligonucleotide probe coated on the wells of a microtitre plate. The plates were

then washed and incubated with an avidin-HRP (horse radish peroxidase) conjugate. Substrate was added after another wash, colour development was allowed to proceed in the dark, stop solution was added and the O.D. read at 450nm. A sample was considered positive if the O.D. reading was >0.4 O.D. units.

7.3.4. RESULTS

Using all three methods (A: guanidine thiocyanate, B: proteinase K and C: the Roche Amplicor kit) 3 HCV RNA positive samples were detected in the set of 10 donor samples (table 7.2.). Sample no.10 was determined by the Eurohep group to be a weak positive, and failed to be identified by any of the three methods. In set 1 of the 10 fold dilution series, the first 2 dilutions out of 6 were positive using methods A and C, while the third dilution was detected using method B. In set 2, the first dilution only was detected using method A, the second dilution with method B and dilutions 1 and 3 using method C. The third positive sample probably representing a false positive reaction in the Amplicor test (table 7.2.). These results demonstrated a ten fold increase in detection rate using the proteinase K method over the guanidine thiocyanate method.

Further comparison between the " in house " RT-PCR and the Amplicor test kit was made. 14 RIBA-3 confirmed/RT-PCR positive samples were also positive with the Amplicor test. Testing of 9 RIBA-3 indeterminate/RT-PCR positive samples produced 1 Amplicor result that was discrepant. This sample showed reactivity with the c33c antigen (band strength 4+) in the RIBA-3, was RT-PCR positive with the " in house

" PCR and negative in the Amplicor test. The same sample was tested again with both systems and the results confirmed. In addition when 2 follow up samples obtained from the same donor were tested by RT-PCR, both were positive for HCV RNA. Nine out of 10 RIBA-3 confirmed/RT-PCR negative samples produced negative results with the Amplicor test, with 1 sample producing a positive signal. Repeat testing of this sample generated a result that was marginally over the cut-off level (result of 0.411 with a cut-off value of 0.4), suggesting a false positive result in the first round of testing. Similarly, comparison of 15 RIBA-3 indeterminate/RT-PCR negative samples produced 1 sample that gave a positive result initially but on repeat testing it proved to be negative (table 7.3.). All discrepant results were confirmed independently by Dr.B.Dow at Ruchill Hospital, Glasgow, using both PCR systems as described.

Table 7.2. EUROHEP PANEL

Blood Donor Sample No.	Result of Method A	B	C	Amplicor O.D.
1	N	N	N	0.143
2	N	N	N	0.125
3	N	N	N	0.123
4	P	P	P	2.372
5	N	N	N	0.152
6	P	P	P	2.181
7	N	N	N	0.121
8	N	N	N	0.126
9	P	P	P	2.214
10	N	N	N	0.132
Log.dil./Series				
-2/1	P	P	P	2.384
-3/1	P	P	P	2.001
-4/1	N	P	N	0.170
-5/1	N	N	N	0.128
-6/1	N	N	N	0.129
-7/1	N	N	N	0.133
-2/2	P	P	P	2.220
-3/2	N	P	N	0.116
-4/2	N	N	P	1.443
-5/2	N	N	N	0.118
-6/2	N	N	N	0.153
-7/2	N	N	N	0.169

P : Positive N : Negative

Cut-off value for Amplicor O.D. readings : 0.4 units

A : Guanidine Thiocyanate method
B : Proteinase K method
C : Roche Amplicor method

Table 7.3.
Comparison of RT-PCR with Amplicor

Sample I.D.	RIBA-3 Result	RT-PCR Result	Amplicor Result	Amplicor O.D.
RIBA-3 CONFIRMED/RT-PCR POSITIVE				
13067	4-4-4-4	P	P	2.354
13069	4-4-4-4	P	P	2.342
13139	4-4-4-0	P	P	2.256
13177	4-4-4-0	P	P	2.231
13179	4-4-4-0	P	P	2.252
13436	1-3-4-0	P	P	2.659
13515	0-3-4-0	P	P	2.598
13669	4-4-4-4	P	P	2.598
13722	4-4-4-3	P	P	2.618
13773	4-4-4-4	P	P	2.566
15038	4-4-4-4	P	P	2.199
15048	0-4-4-4	P	P	2.130
15259	4-4-4-0	P	P	2.361
14888	1-3-4-0	P	P	0.862
RIBA-3 INDETERMINATE/RT-PCR POSITIVE				
14827	0-4-0-0	P	N	0.080*
14993	0-2-0-0	P	P	2.365
15593	0-4-0-0	P	P	2.187
10802	4-0-0-0	P	P	2.673
10886	0-0-4-0	P	P	2.618
8514	0-0-4-0	P	P	2.691
5812	0-0-4-0	P	P	2.666
5519	0-0-4-0	P	P	2.607
4849	0-0-4-0	P	P	2.375

* Repeat test O.D. : 0.070

Order of RIBA-3 antigens : c100-3-c33c-c22-3-NS5 : numbers refer to band strength

Table 7.3.continued
Comparison of RT-PCR with Amplicor (continued)

Sample I.D.	RIBA-3 Result	RT-PCR Result	Amplicor Result	Amplicor O.D.
RIBA-3 CONFIRMED/RT-PCR NEGATIVE				
13229	0-1-3-0	N	N	0.083
13279	0-1-2-0	N	N	0.122
13858	4-4-3-0	N	P	1.290**
13995	4-4-4-0	N	N	0.132
14484	3-4-4-2	N	N	0.125
15215	2-3-3-2	N	N	0.066
15455	0-2-2-0	N	N	0.076
15992	0-1-1-0	N	N	0.063
16056	4-4-4-4	N	N	0.054
13241	0-0-2-1	N	N	0.061
RIBA-3 INDETERMINATE/RT-PCR NEGATIVE				
12065	0-3-0-0	N	N	0.101
13055	0-0-0-1	N	P	1.432***
13065	0-1-0-0	N	N	0.136
13084	0-0-3-0	N	N	0.136
13116	0-0-0-2	N	N	0.124
13118	2-0-0-0	N	N	0.125
13122	2-0-0-0	N	N	0.152
13129	0-0-0-1	N	N	0.125
13149	0-0-0-2	N	N	0.120
13173	0-0-1-0	N	N	0.148
13101	0-0-0-2	N	N	0.070
14926	0-0-1-0	N	N	0.160
15199	4-0-0-0	N	N	0.130
15218	0-0-0-3	N	N	0.154
15287	0-2-0-0	N	N	0.127

** Repeat test O.D. : 0.411
*** Repeat test O.D. : 0.058

7.3.5. DISCUSSION

Detailed analysis was made by the Eurohep group of the results from 31 laboratories on the supplied panel samples. Their results revealed that only 5 (16%) of the participants generated perfect results in testing the blood donor panel and amongst these 5 laboratories the sensitivity of detection in the dilution series varied by a factor of 100. False negative results were mainly due to a lack of sensitivity, failure to detect a weak positive sample in the blood donor panel was found along with decreased detection in the dilution series. However 4% of the tests of strong positive samples were reported as being negative. The rate of false positivity was even greater at 11% of tests of negative samples, with contamination being the most likely explanation for such results. The main conclusions derived from the study was that contamination was a significant problem in some laboratories and that incorporation of negative control samples would help to show if this occurred. The lack of sensitivity in detection by some laboratories could be assessed by the inclusion of a weak-positive control on a routine basis.

In the work from this laboratory, no false positive results were generated in testing the Eurohep panel and the proteinase K digestion method provided a tenfold increase in sensitivity over the guanidine thiocyanate method (table 7.2.: A and B) giving a sensitivity comparable with that of laboratories with good results in the dilution series. However the weak positive sample in the blood donor panel was not detected by any of the 3 methods used here, a problem shared by 10 (33%) other laboratories. It is important to establish a level of sensitivity for the RT-PCR system used and to

monitor the sensitivity. These findings have been incorporated into our routine PCR testing; the proteinase K extraction method is now used and sensitivity is measured by testing five 10-fold dilutions of a HCV RNA positive plasma sample along with any test samples (chapter 2, section 2.7.). Any variation in the end point detection would indicate a decrease in the sensitivity of that set of test samples and the need to retest the complete sample set.

Forty-eight Scottish blood donor samples with different strength and combination of RIBA-3 band were selected to examine whether there was any particular RIBA-3 pattern that failed to be detected by either system and to compare the results obtained. Forty-five samples produced identical results when tested with the " in-house " RT-PCR and the Amplicor kit (table 7.3.). Of the 3 discrepant results, 1 (sample I.D.: 14827) appeared to be a false negative result while the other 2 are likely to be false positives (sample I.D.s: 13858 and 13055)(table 7.3.). A trend that emerges from these results is that a genuine positive result with the Amplicor test produces an O.D value of >2.0 and so it may be possible to identify potential false positive results by their low but positive O.D.. This trend is supported by the false positive sample detected in the second dilution series of the Eurohep panel where the third dilution sample gave an O.D. value of 1.443 (false positive) and the previous dilution had generated a value of 0.116 (negative result).

The Amplicor kit provides a step towards automation of the PCR. Results for ninety six samples could easily be obtained in one day by one person whereas using

conventional techniques it takes approximately 2.5 days or longer (Jackson, 1990) to obtain results on 48 samples. However care must be taken in interpreting the results as false positive and negative reactions do occur.

7.3.6. APPENDIX TO SECTION 7.3.

A second Eurohep panel consisting of 26 coded samples (10 blood donor samples and 2 dilution series) were tested in this laboratory in 1993 using the proteinase K extraction method. The results were recently made available (June 1994) to the participants. A full analysis comparing the results from this laboratory and other participants will be made by the Eurohep group as before, but is not yet available. Table 7.2a shows the results obtained in this laboratory compared with two reference laboratories (ref.lab.) selected by the Eurohep group. Three positive blood donor samples were detected by all three laboratories, ref.lab.1 reported a weakly positive sample not detected by ref.lab.2 or this laboratory. In the first dilution series, the results from this laboratory were the same as ref.lab.2 a positive result was detected to the third dilution, ref.lab.1 reported a positive result in the fourth dilution and a +/- result in the fifth. In the second dilution series this laboratory and ref.lab.1 reported positive results to the fourth dilution with a +/- result in the fifth dilution reported by ref.lab.1. In the second series ref.lab.2 reported a positive result to the third dilution. No false positive reactions were reported by this laboratory.

Table 7.2a. EUROHEP PANEL

Blood Donor Sample No.	IH RT-PCR	Ref. Lab 1	Ref. Lab 2
1	N	N	N
2	P	P	P
3	N	N	N
4	P	P	P
5	N	P*	N
6	N	N	N
7	P	P	P
8	N	N	N
12	N	N	N
13	N	N	N
Dil.Series Dil.factor			
100	P	P	P
1000	P	P	P
4000	P	P	P
16000	N	P	N
64000	N	+/-	N
256000	N	N	N
1024000	N	N	N
4096000	N	N	N
10	P	P	P
100	P	P	P
1000	P	P	P
4000	P	P	N
16000	N	+/-	N
64000	N	N	N
256000	N	N	N
1024000	N	N	N

IH RT-PCR : in-house RT-PCR (results from this laboratory)

* reported as a weak positive

7.4. THE ROLE OF PCR AS A VALIDATION ASSAY FOR VIRAL INACTIVATION PROCEDURES

7.4.1. INTRODUCTION

The screening of individual blood donations prior to infusion or incorporation of plasma into a plasma pool used in the manufacture of factor concentrates has proved to be effective at reducing the transmission of infectious agents. To provide an additional level of safety, blood product manufacturers are required to incorporate virucidal inactivation procedures into the manufacturing process (chapter 1, section 1.3.). It is also necessary for manufacturers to provide evidence of the virucidal potential of the virus inactivation methods (chapter 1, section 1.3.1.). Infectivity assays using model viruses are widely used since there is no reliable culture system for the agents HBV, HCV, HDV and parvovirus B19. Such model systems are only a representation of the behaviour of an infective agent. However there is a case for the use of the actual pathogenic agent as the results obtained would more accurately mimic those of the virucidal process. Development of alternative methods for the assessment of virus inactivation would therefore be required. In the following section, RT-PCR was used to detect the presence of viral nucleic acid in blood products and these results were compared with two different infectivity assay systems based on tissue culture to investigate the potential role of PCR in monitoring virus inactivation in production methods using solvent/detergent and heat treatment.

7.4.2. SAMPLES

(1) HIV : HIV-1 (lab strain RF) was spiked at a ratio of 1:20 into intermediate-purity factor VIII and factor IX concentrate and at 1:12 into fibrinogen concentrate produced by standard manufacturing methods used at the PFC (refs in Hart et al. 1993). Spiking with HIV-1 at this ratio does not have any effect on the concentrates during freeze-drying and virus inactivation by heating (pers. comm. Dr.H.Hart, PFC, Edinburgh). Laboratory-scale freeze-driers were used that maintained the same yield, stability and moisture levels during freeze-drying that occur during normal production. Similarly the severe heating of the concentrates was carried out using scaled models of the heat-cycle regimes. Samples were heated to 80^{0}C and two separate vials were removed at various time intervals (24, 48 and 72 hours). One vial was designated for testing by the infectivity assay, the other for testing using RT-PCR. The samples were reconstituted in distilled water and stored at -80^{0}C until testing. The tissue culture assays were carried out by Dr.H. Hart (PFC, Edinburgh) and were based on the formation of syncytia in C8166 cells and described in Hart et al., 1993. RT-PCR was carried out as described in chapter 2, section 2.5., 2.6.. HIV specific primers (Simmonds et al., 1990b) are described in chapter 2, section 2.6., table 2.5. (5′ nucleotide positions relative to HIV clone HXB2).

(2) CANINE PARVOVIRUS : Canine parvovirus (CPV) is commonly used as a model virus for parvovirus B19 (chapter 6) since it is not possible to maintain the latter in culture. CPV was cultured to titres of $10^{4.2}$ $TCID_{50}$ and spiked at a ratio of 1:10 into high purity factor VIII concentrate. Samples were heated, as before, to 80^{0}C

and two separate vials were removed for infectivity assay or PCR testing at various time intervals (8, 24, 48 and 72 hours). The tissue culture assays were carried out by Dr.H. Hart (PFC, Edinburgh) and the presence of virus was detected by haemagglutination of porcine red cells (Hart et al. Vox Sang in press). PCR was carried out using primers derived from a consensus of feline and canine parvovirus sequences (described in chapter 2, section 2.6., table 2.3.; 5′ nucleotide numbered as in Shade et al., 1986).

7.4.3. RESULTS

(1) HIV : Using dry-heat treatment at 80^{0}C the infectivity of HIV-1 was rapidly inactivated (table 7.4.). After 24 hours of heating, infectious HIV-1 was isolated from the factor VIII and factor IX preparation which had been spiked with the higher HIV-1 inoculum, but not from the fibrinogen concentrate (spiked at a lower level). Infectious HIV-1 was not detected in any of the concentrates after 48 or 72 hours of heating.

HIV-1 RNA detected by RT-PCR in the same material was found to persist even after 72 hours of heating at 80^{0}C, and over time the difference between the infectivity and RT-PCR measurements became greater. The virus reduction index for HIV-1 measured by the infectivity assay was calculated as 5.0, 4.0 and 4.5, averaging 4.5 $\log_{10}$ (table 7.4.). The RT-PCR reduction indexes were 2.0, 2.0 and 1.0, averaging 1.7$\log_{10}$ for the three different concentrates. The reduction index measured by RT-PCR was therefore less than that measured by infectivity assay. In addition, 10^5

copies/ml of HIV-1 could be detected by RT-PCR after 72 hours of heating when the infectivity assay failed to detect the presence of infectious HIV-1 particles.

(2) CANINE PARVOVIRUS

Similar results were obtained when PCR were compared to an infectivity assay for the detection of CPV. Virus infectivity was decreased by a factor of $2.1\log_{10}$ after 24 hours heating at 80^0C, and fell below the sensitivity of the assay by 48 hours (table 7.5.). In contrast, there was no reduction in the titre of CPV detected by PCR, 6.8 X 10^8 copies/ml were detected for all time points, except the final time point which was found to be 7.6 X 10^8 copies/ml (this difference is likely to be caused by variation in pipetting and is probably not significant).

Table 7.4.

COMPARISON OF HIV-1 INFECTIVITY ASSAY AND PCR AFTER DRY HEAT-TREATMENT OF FACTOR CONCENTRATES

Dry heat treatment (hours)	Factor VIII		Factor IX		Fibrinogen Conc.	
	HIV-1 infectivity (IVIU/ml)†	PCR (copies/ml)	HIV-1 infectivity (IVIU/ml)	PCR (copies/ml)	HIV-1 infectivity (IVIU/ml)	PCR (copies/ml)
Before freeze-drying	2×10^7	10^9	2×10^7	10^9	2×10^4	10^7
After freeze-drying	2×10^5	10^7	2×10^5	10^7	2×10^4	10^6
80°C						
24 hours	20	10^6	2×10^3	10^7	<20	10^6
48 hours	<2	10^6	<20	10^6	<20	10^6
72 hours	<2	10^5	<20	10^5	<20	10^5
Reduction index* ($\log_{10}$)	5.0	2.0	4.0	2.0	4.5	1.0

* After heating

† *in vitro* infectious units : the last dilution showing HIV-1 replication confirmed by p24 antigen assay.

Table 7.5.

COMPARISON OF CPV INFECTIVITY ASSAY

AND PCR AFTER DRY-HEAT TREATMENT

Dry heat treatment (hours)	Factor VIII	
	CPV Infectivity $\log_{10}$ $TCID_{50}$/ml	PCR (copies/ml)
before freeze-drying	4.2	6.8×10^8
after freeze-drying	4.2	6.8×10^8
80°C		
8 hours	3.3	6.8×10^8
24 hours	2.2	6.8×10^8
48 hours	≤2.1	6.8×10^8
72 hours	≤2.1	7.6×10^8
Reduction index* ($\log_{10}$)	≥2.1	≤0

* After heating

7.4.4. DISCUSSION

In the UK, manufacturers of blood products are required to show that a single step in the manufacturing procedure is capable of inactivating 5 logs of HIV-1 (Thomas et al., 1988). Many different studies have been published on HIV-1 inactivation, assessed by using different virus strains, culture cells and culture times (Prince et al., 1986a,b; Spickett et al., 1989; Tersmette et al., 1986) and with no international standard, comparison of different infectivity assays is difficult. The HIV-1 infectivity assay system used in this work required a 28 day culture time and the presence of virus was assessed by the detection of syncytia and the presence of p24 antigen in the culture supernatant. One potential advantage of RT-PCR over the infectivity assays is that a result would be available within 2 days and quantitation of the number of virus particles could be made. However, the reduction index after 72 hours heat treatment was only $1.7\log_{10}$ measured by RT-PCR compared with $4.5\log_{10}$ by infectivity assay. Factor concentrate treated by heating to 80^0C has been evaluated in patient trials (Watson and Ludlam, 1992) and has been found to be effective based on the prevention of transmission of HIV and HCV to haemophiliacs (Haemophilia Directors for Scotland and Northern Ireland, 1993). The results of this study suggest that heat stable RNA sequences, either intact or in fragments, can remain in noninfective HIV samples.

A similar trend was observed on comparison of the results from the CPV infectivity assay and the PCR results. PCR positive results were obtained from samples which had no demonstrable infectivity in tissue culture. The use of solvent detergent as a

virus inactivation procedure is primarily targeted at the elimination of lipid-enveloped viruses, however terminal dry heat treatment of solvent detergent treated factor concentrate is effective at inactivating the non-lipid enveloped virus HAV (Hart et al., Vox Sang in press). Care must be taken in interpreting the CPV results since transmission of human parvovirus B19 by factor concentrates has been shown to occur with dry heat treated preparations (Bartolomei-Corsi et al., 1988; Lyon et al.,1989). Parvovirus B19 has also been transmitted to haemophiliacs despite the terminal heating to 100^{0}C for 30 min of solvent/detergent treated concentrates (Santagostino et al., 1994). The results presented here based on the CPV model indicate that a longer period of heating (at least 24 hours) might be necessary to inactivate virus. Although analogies can be drawn between CPV and parvovirus B19, an *in vitro* system for B19 is necessary before a full assessment of inactivation procedures can be made.

In conclusion, the poor correlation between the RT-PCR results and the infectivity assay for both HIV-1 and CPV indicates that RT-PCR is probably not useful for the evaluation of virus inactivation methods. The results obtained in this study along with clinical evidence of parvovirus B19 transmission to haemophiliacs suggests that alternative inactivation strategies are necessary to eliminate the risk of parvovirus B19 transmission. Preliminary studies have been carried out using ultraviolet light treatment (UV A and UV C) in the presence of psoralens (see chapter 1, section 1.3. table 1.5.) on factor VIII concentrates spiked with parvovirus B19. Titration of parvovirus B19 DNA before and after treatment showed 3 log reduction in titre after

treatment (data not shown, work carried out in collaboration with Dr.H. Margolis-Nunno, NYBDC, New York). This UV light/psoralen treatment may provide a supplementary measure to standard manufacturing processes that will help improve the safety of blood products.

CHAPTER 8

8. DISCUSSION

The aim of this thesis is to make a contribution through the use of PCR to the improvement of the safety of blood and factor concentrates for clinical use. At present safety depends on the selection of donors through interview and screening for virus specific antibody or antigen in an attempt to prevent a viraemic unit from being infused and reduce the virus load in a plasma pool prior to production of factor concentrates. The cost of withdrawing a contaminated pool once it has gone into production would be in the region of £100,000 to £1,000,000 (SNBTS unpublished data). The benefits of identifying contaminated donations prior to use, from the aspect of safety and monitory saving, are great. In addition, validated virus inactivation procedures are required in the production procedure before factor concentrates are licensed, providing a further level of safety. The properties of a virus that would indicate potential transmission by blood or blood products are (a) those that persist in the blood and at high titre; (b) those that have a long incubation period before the development of symptoms and (c) those that are mild or asymptomatic infections. The viruses considered in this thesis, namely HCV, parvovirus B19 and HAV, each exhibit some of these characteristics, causing different problems clinically and in the field of blood transfusion.

For HCV infection, there may be a long window period (6-12 months or longer), in which an individual is viraemic but asymptomatic. Viraemia is generally low (average titre 10^5 copies /ml), but usually results in chronic infection. The ultimate severity of the disease can be life threatening, early diagnosis of infection is

important not only from the transfusion microbiologists point of view but for the individual also. Anti-HCV screening can detect most infected individuals. However a considerable number of EIA positive samples produce an indeterminate result (38.2%, this study) in RIBA-2 screening, a proportion of which (5.5%, this study) are viraemic. Exclusion of all indeterminate donations from further use would be costly and wasteful and so further confirmatory tests are required. Identification of viraemia by RT-PCR was the approach taken in this work and proved effective at confirming HCV viraemia in antibody positive and indeterminate donations. It was also demonstrated that different HCV genotypes influence the serological response elicited. Infection with HCV type 1 showed broad reactivity with antigens in RIBA-2, while infection with other genotypes (2-6) elicited antibody responses restricted to two antigens only (c22-3 and c33c). This observation provides an explanation for the continued transmission of HCV by blood screened with assays that relied in reactivity to one antigen alone (c100-3). These results also indicate that infection with different HCV genotypes will be detected with the currently available 2nd and 3rd generation assays since reactivity with the c22-3 antigen was detected in all RIBA-2 positive, PCR positive donors tested and in 92% of RIBA-2 indeterminate, PCR positive donors. HCV type-specific PCR based and serological assays are currently being developed (van Doorn et al., 1994, Simmonds et al., 1993a). An ideal assay would be one that is equally sensitive for the six different genotypes. The importance of determining HCV genotype probably lies in the clinical situation since different genotypes may vary in their infectivity, pathogenicity and response to interferon treatment (Dusheiko et al., 1994). Preliminary data suggests that it may be important

to identify subtypes of the different genotypes as individuals infected with HCV type 1a show a better response to interferon treatment than those infected with 1b (Takada et al., 1992b; Yoshioka et al., 1992; Kanai et al., 1992). Conserved polymorphisms in HCV subtypes 1a, 1b, 2a, and 2b sequences have been identified, and the RFLP system described in this thesis could be extended in order to differentiate these subtypes.

The major benefits derived from use of the PCR in the blood transfusion setting is the ability to detect virus in the " window period " or seronegative stages of infection and for the detection of viruses in products made from large pools of plasma. The techniques described in this thesis could be used to supplement serological screening for other transfusion-transmitted viruses. For example, hepatitis B virus transmission still occurs despite routine screening for hepatitis B surface antigen and might be eliminated by screening donations for HBV DNA in pools. Another significant problem is infection with human cytomegalovirus (HCMV) via blood transfusion to immunocompromised individuals, such as transplant recipients. HCMV detection by PCR can be performed more quickly and with greater sensitivity than conventional serological techniques (Bevan et al., 1991). PCR can also be used for the detection of bacterial pathogens. Transfusion of erythrocytes contaminated with the bacteria *Yersinia enterocolitica* can cause sepsis with high mortality rates (Blumberg et al., 1991). Asymptomatic blood donors are recognised as the source of transfusion endotoxemia caused by *Y. enterocolitica*. Conventional methods for identifying the bacteria require lengthy incubations of cultures (Feng et al., 1992; Kwaga et al.,

1992) and could be replaced by PCR. There have also been reports of blood donors who later developed Creutzfeldt-Jakob disease (CJD)(Esmonde et al., 1993). As yet, transmission of CJD has not been linked to blood transfusion, and little is known about the incubation period or nature of the infectious agent. Clearly identification of the agent would be desirable, if it is found to be a virus the application of PCR would be possible.

The application of the PCR in detecting virus nucleic acid remains largely a research tool as it is considered too costly, time consuming and difficult to standardise for use on a routine basis. In the work described in this thesis it has been attempted to carry out PCR testing in a timescale and budget that would enable viraemic donations to be withdrawn prior to use. For HCV the approach was to use RT-PCR to augment and confirm the results of antibody screening. Two other viruses, parvovirus B19 and HAV could potentially contaminate plasma pools or be transmitted through infusion of infected donations. In adults infection is generally acute and asymptomatic in the case of B19, viraemia precedes any symptoms and high virus titres can be attained in infected individuals. At present, routine donor screening for B19 or HAV is not carried out. Using a pooling system it was possible to screen a large number of donations for the presence of contaminating virus and in addition provide information on the epidemiology of infection.

While the pooling technique used in this thesis offers one approach to screening a large number of donations, other techniques are available. The Roche Amplicor HCV

PCR kit (described in chapter 7, section 7.3.3.) is a PCR based assay that has a colorimetric readout. This similarity to ELISAs may be more attractive to Blood Transfusion Service Departments in which staff are accustomed to microtitre plate formats. Kits are also available for the detection of HIV and CMV. Another approach for rapid detection of HCV has been based on the direct capture of HCV RNA from plasma (van Doorn et al., 1994). HCV-RNA is hybridised to a complementary biotinylated oligonucleotide, followed by capture of the resulting hybrid onto streptavidin-coated beads followed by cDNA synthesis and PCR allowing for the detection of (near) full length HCV RNA. Both of these assays have eliminated the phenol/chloroform extraction and ethanol precipitation of nucleic acid and so have greatly speeded up the processing of samples. A non-PCR based assay developed by Chiron Corporation requires no sample preparation. A crude proteinase K lysate of RNA is hybridised in solution to two sets of oligonucleotide probes one of which is captured onto a solid phase. The other oligonucleotide hybridises to a branched DNA molecule (bDNA) which serves to amplify the signal by binding multiple copies of an enzyme linked oligonucleotide. A positive result is indicated by a chemiluminescent signal (Urdea, 1993). Similar assays are available for HBV, HIV and CMV. This assay is quantitative but is limited in its sensitivity. If the sensitivity of the assay can be enhanced it would find widespread application in the field of blood transfusion.

A criticism of the PCR has been the generation of false positive results due to contamination with product from previous reactions. This can be overcome by having

separate laboratories and designated equipment for each stage, from extraction, cDNA reaction, primary PCR, secondary PCR to running the product on an agarose gel. An additional measure taken by some laboratories is the use of uracil in place of thymidine in the PCR reaction mix. The uracil becomes incorporated into amplified sequences in subsequent reactions any amplified material from previous reactions is destroyed by the enzyme uracil N-glycosylase before the next rounds of cycling. The length of time taken in setting up reactions may be reduced with the advent of new thermostable DNA polymerase enzymes now available that can be used in place of Taq. *Thermus thermophilus* (Tth) offers the advantage of having reverse-transcriptase properties in addition to polymerising activity and can therefore be used to carry out both reactions in the one tube (Young et al., 1993) providing not only a saving in time but a reduction in the possibility of contamination. The use of uracil and Tth enzyme are both used in the Roche Amplicor Kit.

That blood products can be the source of virus transmission was demonstrated using RT, PCR and direct sequence analysis, HAV, B19 and HCV sequences could be detected in factor concentrates and/or intravenous immunoglobulin preparations. Sequence analysis detected similarities between HCV sequences obtained from recipients and contaminated batches of IVIG, and phylogenetic analysis demonstated a link with the source of infection. PCR and other virus nucleic acid detection techniques will undoubtably have a role in improving the safety of blood and blood products in the future. Routine screening of blood donations, plasma pools and blood products for viruses is recognised by transfusion centres worldwide as the next step

in improving the safety of blood and its products.

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